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Immunostimulating Activity of Polysaccharides from Mycelia of *Phellinus linteus* Grown under Different Culture Conditions

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Polysaccharides were extracted from mycelia of *Phellinus linteus* grown under different culture conditions. The *in vitro* immunostimulating activity was measured by plaque-forming cell (PFC) assay. The activity of the polysaccharides was different from that of mycelia from which was extracted. The number of PFC's ranged from 40 to 600 depending on the media. When *P. linteus* was cultured on a medium with mannose or starch as a sole carbon source, the fungus produced polysaccharide with the highest activity of 960 PFC. Activity was therefore increased by 50% compared with polysaccharide which was extracted from mycelia grown on medium with glucose. pH had little effect on the change in activity. All polysaccharides on media with different pH stimulated about 600 PFC. These results suggest that activity could be increased by polysaccharide modification through changes in physiological conditions.

Numerous polysaccharides from different biological origins, e.g., yeast, algae, bacteria, higher plants, and especially fungi have been investigated for antitumor and immunomodulating activities (6). The active polysaccharides showed antitumor effects against allogenic, syngenic, and even autologous tumors. Most antitumor polysaccharides have been isolated from basidiomycetes. β-(1-3)-glucans were known to be the most efficient. Lentinan (1), Schizophyllan (13), Krestin (14), and Meshima (7) are now in clinical use. Song *et al.* (12) reported that a polysaccharide from the mycelia of *Phellinus linteus* stimulated polyclonal antibody production *in vitro*. Moreover, the polysaccharide stimulated immune functions of T lymphocytes and nonspecific immune functions mediated by natural killer cells and macrophages (8).

Polysaccharide modification and improved production have been made by chemical, enzymatic, and physiological methods, and also by the application of genetic engineering technology. In case of antitumor polysaccharides, many chemical modifications have thus been made to improve the activity (2, 9). Few studies, however, have been made regarding the modification and production improvement of the polysaccharide through physiological methods. Previously we reported the modification of polysaccharide from mycelia of *P. linteus* grown under different culture conditions (10). In this paper, the immunostimulating activity of the modified polysaccharide was studied.

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MATERIALS AND METHODS

Preparation of Inoculum

Phellinus linteus L13202, which was obtained from the United States Department of Agriculture, was used and preserved on a PDA (potato 200 g, dextrose 20 g, agar 20 g per liter) plate. Ten pieces of 4×4 mm solid media from the PDA were put into 100 ml YMG (yeast extract 4 g, malt extract 10 g, glucose 4 g per liter) broth in a 250 ml Erlenmeyer flask. The broth was cultured at 30°C on a rotary shaker for 10 days and homogenized at 10,000 rpm for 3 min. The 10 ml of homogenate was used as an inoculum.

Culture Condition

The fungus was cultivated on different media: GPY (glucose 20 g, polypeptone 2 g, yeast extract 2 g, $NaNO_3$ 3 g, $NaHPO_4$ 10 g, KCl 0.5 g, $MgSO_4$ 0.5 g per liter); PYG (peptone 1.25 g, yeast extract 1.25 g, glucose 14 g per liter); YM (yeast extract 4 g, malt extract 10 g); CP medium (carbon source 30 g, peptone 3 g, K_2HPO_4 10 g, $MgSO_4$ 0.7 g per liter). In studies of the carbon source

effect on activity, CP medium containing 3% (w/v) of the carbon source was used. For pH experiments, the pH of the CP medium with glucose as a carbon source (CPG) was adjusted from 5 to 9 with 1 N HCl or 1 N NaOH after autoclaving. Three flasks were prepared for each carbon source and pH experiment. The fungus was grown for 12 days on a rotary shaker at 30°C.

Isolation of Polysaccharide

After cultivation for 12 days, the mycelia were harvested by filtration, suspended in an appropriate amount of water and homogenized at 15,000 rpm for 3 min. The homogenate was heated at 100°C for 1 h with refluxing and then centrifuged (5,000 rpm × 5 min). The supernatant was saved. The pellet was resuspended in water and the suspension was subjected to boiling again. This procedure was repeated two more times. All three supernatants were combined and treated with ethanol to 80% (v/v). The mixture was stood at 4°C overnight and centrifuged. The polysaccharide pellet was dissolved in water and dialyzed for 2 days. The non-dialyzable fraction was freeze-dried to produce the polysaccharide.

***In vitro* Immunostimulating Activity**

The *in vitro* T-independent and polyclonal antibody response was determined by plaque forming cell (PFC) assay using sheep red blood cell as previously described (12). Briefly, lymphocyte cells from a specipic pathogen-free mouse spleen were suspended in RPMI 1640 with 10% fetal calf serum and adjusted to 0.5×10^7 cells/ml. Polysaccharide sample was added at a final concentration of 100 μg/ml. The polyclonal antibody response was measured after *in vitro* stimulation for two days. The number of PFC's was counted by a haematocytometer.

Gel Permeation HPLC

Polysaccharide was dissolved in water at a concentration of 1 mg/ml. An 10 μl aliquot of the solution was injected into a Tosoh GMPW column (7.8 × 300 mm). HPLC conditions were as follows: column temp., 25°C; detection, refractive index; mobile phase, water; flow rate, 1 ml/min.

RESULTS AND DISCUSSION

Several different media, which have been used for the cultivation of fungi and mushrooms, were tested in order to select a medium which yielded a good production of polysaccharide. Polysaccharide was extracted from mycelia which had been grown on different media and tested for immunostimulating activity. Fig. 1 shows that polysaccharides from mycelia grown on YMG and CPG had the higher activity. The polysaccharide from mycelia which were grown on CPG medium stimulated about 630 PFC. On other media, the fungus did not produce polysaccharide with a high degree of activity. Among media tested, the CPG medium also gave good mycelial growth and polysaccharide production (10). Thus, CP medium was selected for further study.

After the fungus was cultured on CP medium with different carbon sources, the polysaccharide was extracted from each mycelium. The polysaccharide showed different levels of immunostimulating activity (Fig. 2). Thus, mycelia which was grown on mannose or starch produced polysaccharide with the highest activity. The polysaccharide stimulated around 1,000 B lymphocyte

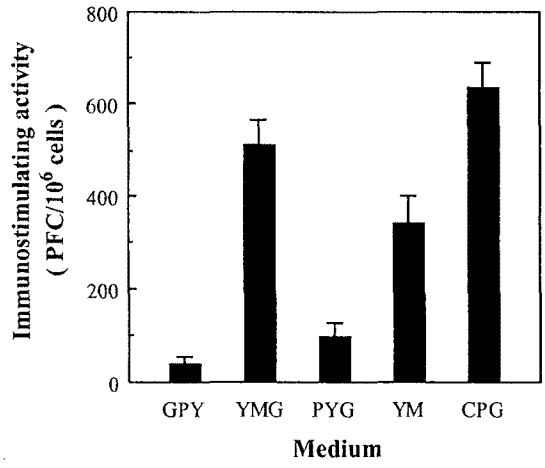

Fig. 1. Immunostimulating activities of polysaccharides from mycelia of *P. linteus* which were grown on different media.
The used media are followings: GPY (glucose 20 g, polypeptone 2 g, yeast extract 2 g, $NaNO_3$ 3 g, $NaHPO_4$ 10 g, KCl 0.5 g, $MgSO_4$ 0.5 g per liter); YMG (yeast extract 4 g, malt extract 10 g, glucose 4 g per liter); PYG (peptone 1.25 g, yeast extract 1.25 g, glucose 14 g per liter); YM (yeast extract 4 g, malt extract 10 g); CPG (glucose 30 g, peptone 3 g, K_2HPO_4 10 g, $MgSO_4$ 0.7 g per liter).

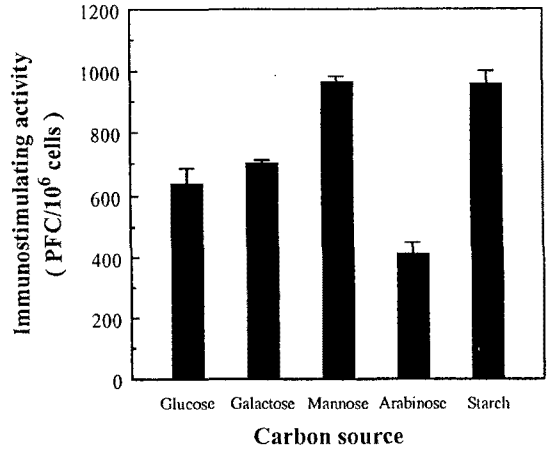

Fig. 2. Immunostimulating activities of polysaccharides from mycelia of *P. linteus* which were grown on CP medium with different carbon sources.
The composition of the used CP medium is following: carbon source 30 g, peptone 3 g, K_2HPO_4 10 g, $MgSO_4$ 0.7 g per liter.

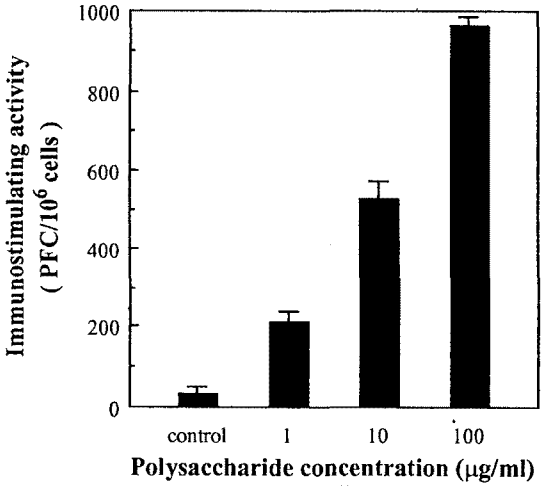

Fig. 3. Dose-dependent immunostimulating activity of polysaccharides from mycelia grown on CP medium with mannose.
The composition of CP medium with mannose is followings: mannose 30 g, peptone 3 g, K_2HPO_4 10 g, $MgSO_4$ 0.7 g per liter. Lymphocyte cell suspension was prepared from a SPF mouse spleen and adjusted to 0.5×10^7 cells/ml. Polysaccharides (1 to 100 μg/ml) were added to the cell suspension for activation of B cell. The number of plaque forming cell (PFC) was counted.

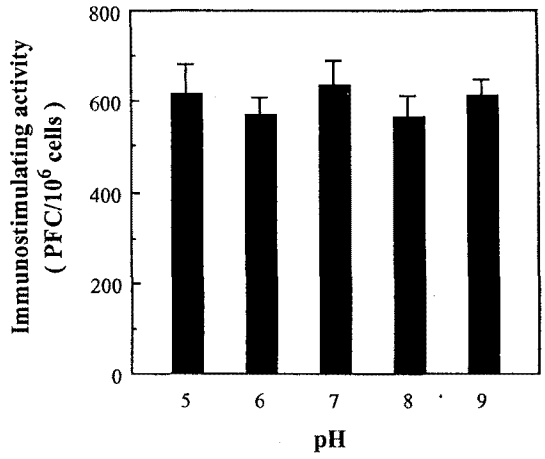

Fig. 4. Immunostimulating activity of polysaccharides from mycelia of *P. linteus* which was grown on CPG medium with different pHs.

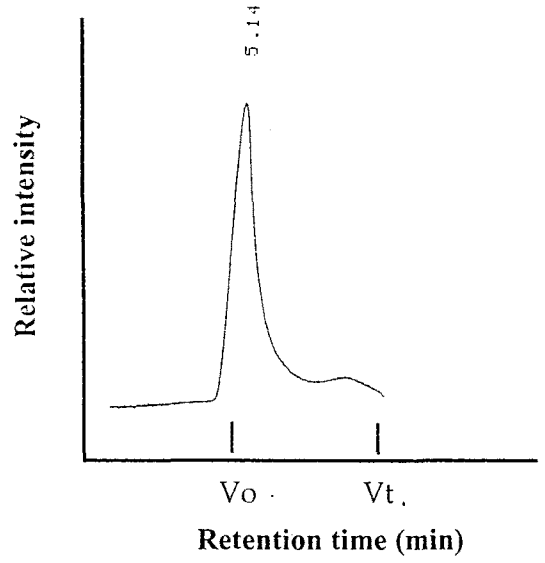

Fig. 5. The gel permeation HPLC elution profile of polysaccharide from mycelia which were grown on CP medium with mannose as a carbon source.
HPLC conditions were as follows: column, Tosoh GMPW (7.8×300 mm); column temp. 25°C; detection, refractive index; mobile phase, water; flow rate, 1 ml/min.

cells to produce antibodies. Fig. 3 shows that the response also was dependent on the concentration of polysaccharide applied. Arabinose as a carbon source was not good with regard to the activity. When *P. linteus* was grown on CP medium with different carbon sources, the extracted polysaccharide had a similar monosaccharide composition. However, the molar ratio of each monosaccharide was different to each other (10). The difference in activity might thus reflect in the difference of its structure. Previously, Domer *et al* (4) reported that mannan from *C. albicans* caused 140 antibody production cells per 10^6 cells. Mannan was known to be somewhat less effective than glucan, suggesting that glucan is, in general, a more potent immunostimulator. This fact suggests that the polysaccharides with the higher activity from *P. linteus* may be a glucan.

All polysaccharides extracted from mycelia grown on CPG media with different pHs showed similar activity (Fig. 4). Thus, pH seemed to have little effect on the activity change of the polysaccharide. Previously, we reported that the pH effect on the ratio of monosaccharide in the polysaccharide was not as important as the carbon source. In the case of other basidiomycetes, pH had little effect on antitumor activity, either (5). Therefore, pH seems to have litte effect on the structure of polysaccharide.

It has been reported that the size and branching of fungal polysaccharide were different depending on the growth medium (11). The structure and molecular weight of polysaccharides has been found to be important for immunostimulating activity (3). Thus, the molecular weight of polysaccharides from mycelia which were grown on CP medium with mannose or arabinose as a carbon source was estimated. Fig. 5 showed the elution profile of polysaccharide from mycelia grown on medium with mannose. The molecular weight of the polysaccharide was measured to be about 1×10^6 D. The polymers from mycelia grown on medium with arabinose or glucose

were found to have a similar size (data not shown).

While many studies on the physiological modification of polysaccharide structures have been carried out on microbial exopolysaccharides (15), the approach has not been applied to mycelial polysaccharides. The ongoing results suggest that the immunostimulating activity of polysaccharides can be modified through changes in the physiological conditions for the growth of the fungus. Since all extracted polysaccharides had a similar monosaccharide composition and size, change in activity levels might thus result from the difference in polysaccharide structure such as the linkage type and the degree of branching.

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