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Immunomodulating Activity of the Exopolymer from Submerged Mycelial Culture of *Phellinus pini*

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Abstract The immunomodulating activities and chemical characteristics of a water-soluble exopolymer from submerged mycelial culture of *Phellinus pini* were studied. Anticomplementary activity of this polymer was found to be 73.2%, and its activation system occurred through both classical and alternative pathways, where the classical pathway was detected to be the major one by crossed immunoelectrophoresis. Nitric oxide (NO) release ability and acid phosphatase activity of macrophage were increased by 1.6-fold (100 μg/ml) and 3.4-fold (500 μg/ml), respectively, and splenocyte proliferation in mixed lymphocyte reaction (MLR) was also increased by 2.6-fold (200 μg/ml), compared to the control. The molecular weight of this polymer, determined by HPLC, was under 5 kDa. Total sugar and protein contents were 89.7 and 10.3%, respectively. Both sugar and amino acid compositions of the exopolymer were also analyzed.

Key words: Anticomplementary activity, exopolymer, macrophage activity, *Phellinus pini*, splenocyte proliferation activity

Among various immune systems, the complement system consisting of a series of enzymes in blood serum plays an important role in host resistance as a primary humoral mediated antigen-antibody reaction. Also, the complement system is involved in the induction and regulation of specific immune responses; for example, activation of macrophages and lymphocytes, localization and retention of antigen in germinal centers, generation of B cell memory, cellular cooperation, and regulation of antibody production.

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Recently, some pharmacological activities, anticomplementary activity, mitogenic activity on lymphocytes, interferon-inducing activity and antitumor activity have been observed in polymers such as polysaccharides isolated from several food sources such as hot red pepper [25], *Paeonia moutan* [23] Chinese medicinal herbs such as *Angelica acutiloba* [13], and *Bupleurum falcatum* [2], and mushrooms such as *Grifora umbelata* [7], *Poria cocos* [25], *Ganoderma lacidum* [34], and *Coriolus versicolor* [31]. It was also reported that some exopolymers from the culture broth of submerged mycelial cultures of *Cordyceps militaris*, *Pleurotus ostreatus*, *Ganoderma lucidum*, and *Trametes suaveolens* have high anticomplementary activities when screened for twenty-one kinds of mushrooms in our laboratory [27]. Therefore, it was felt that a more systematic study is necessary to clearly identify the modulation of immune systems.

Our preliminary investigations showed that the exopolymer from culture broth of *Phellinus pini* had high anticomplementary activity. In the present study, several immunomodulating activities such as macrophage activation, and splenocyte proliferation by this polymer were investigated along with its chemical analyses.

Materials and Methods

Strain and Culture Medium

Phellinus pini, obtained from the Rural Development Administration in South Korea, was grown in potato/dextrose broth on a rotary shaker (120 rpm) at 25°C. After 10 days, 100 ml of culture broth was aseptically homogenized and inoculated at 1% (v/v) into culture medium with the following composition (g/l): glucose 20, $MgSO_4$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, yeast extract 2, and

peptone 2; the pH was adjusted to 5.0 before sterilization. Culture broth was harvested by centrifugation (10,447 ×g/ 20 min) and the supernatant was mixed with 80% final concentration of ethanol. Ethanol precipitate was collected, dialyzed against distilled water, and lyophilized to obtain an exopolymer.

Animals

Six-weeks-old C57BL/6 and BABL/c male mice weighing approximately 25 g were purchased from Daehan Biolink Co., Ltd., and were housed in plastic cases divided into 5 groups. The animal room was maintained at constant temperature (22°C±2) and humidity (55%±5) with a 12 h cycle of light and dark.

Reagents and Chemicals

Hemolysin-sensitized sheep erythrocytes (EA cell) were purchased from Lyophilizate Laboratory Co., Ltd. (Japan). Fetal Bovine Serum (FBS), Rosweil Park Memorial Institute-1640 (RPMI-1640) medium, penicillin-streptomycin, and amphotericin B were from GIBCOBRL (U.S.A.). HEPES, Hank's balanced salts solution (HBSS), Triton X-100, $NaNO_2$, Lipopolysaccharide (LPS, *E. coli* 0127:B8), anti-human complement C3, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., Ltd. (U.S.A.). Mouse Interferon-γ (IFN-γ) was purchased from Roche Ltd. (Germany). Dialysis Tubing (MW cut-off; 6,000- 8,000 Da) was purchased from Spectrum Laboratories, Inc. (U.S.A.). All other chemicals and solvent were of analytical grade.

Anticomplementary Activity Assay and Determination of the Complement-Activating Pathway

Anticomplementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement [11]. Fifty microliter of water solution of polymer was mixed with equal volumes of normal human serum (NHS) and GVB (gelatin veronal buffered saline, pH 7.4) containing 500 μg Mg^{++} and 150 μg Ca^{++}. The mixtures were incubated at 37°C for 30 min and the residual total complement hemolysis (TCH_{50}) was determined by using IgM hemolysin sensitized sheep erythrocytes at 1×10^8 cells/ ml. At the same time, the NHS was incubated with deionized water and GVB^{++} (GVB containing 500 μg Mg^{++} and 150 μg Ca^{++}) as a control. The anticomplementary activity of crude polymers was expressed as the percentage inhibition of the TCH_{50} of control.

$$\text{Inhibition of } TCH_{50}(\%) = \frac{TCH_{50} \text{ of control} - TCH_{50} \text{ of treated sample}}{TCH_{50} \text{ of control}} \times 100$$

Ca^{++} is required for the activation of complement *via* the classical pathway, but not the alternative pathway, and the activation through the alternative pathway was measured in Ca^{++}-free condition. Alternative complement pathway was determined in 10 mM EGTA containing 2 mM $MgCl_2$ in GVB^{--} (Mg^{++}-EGTA-GVB^{--}) by a modified method [24]. A sample was incubated with Mg^{++}-EGTA-GVB^{--} and NHS at 37°C for 30 min, and the residual complement mixtures were measured by hemolysis of rabbit erythrocytes (5×10^7 cells/ml) incubated with Mg^{++}-EGTA-GVB^{--}.

Crossed Immunoelectrophoresis

The specific activation of C3 complement component by polymer in NHS was assessed by comparative measurements of C3 cleavage [14]. NHS was incubated with an equal volume of the solution of polymer in GVB^{++} and GVB^{--} containing 10 mM EDTA (EDTA-GVB^{--}) or Mg^{++}-EGTA-GVB^{--} at 37°C for 30 min. The mixture was then subjected to crossed immunoelectrophoresis to locate the C3 cleavage product. All the samples (10 μl) were subjected to isoelectric focusing on 1% agarose gel. Two hours after the first run in barbital buffer (pH 8.6), ionic strength 0.025 with 1% agarose, the second run was carried out in a gel plate, containing anti-human complement C3 to recognize both C3 and C3b, at a potential gradient of 15 mA/plate for 15 h. After the electrophoresis, the plate was fixed and stained with 0.2% bromophenol blue. The ratio between the heights of the C3 and C3b peaks was calculated.

Preparation of Macrophage

Macrophages were harvested from mice three days after an intraperitoneal (i.p.) injection of 3 ml of 10% thioglycolate medium. Cell density was adjusted to 1×10^6 cell/ml with HBSS buffer, supplemented with 10% fetal bovine serum. Afterward, each well of a 96-well microplate was inoculated with 200 ml of the cell suspension (2×10^5 cell/ well). Adherent macrophages were isolated by incubating the cells for 2 h at 37°C, 5% CO_2, and then, vigorously shaking the plate to wash and remove non-adherent cells three times. Cultures were maintained with or without addition of test exopolymers, and in the absence or presence of IFN-γ (20 U/ml) or LPS (the same concentration as exopolymers) at 37°C and 5% CO_2 in a humidified incubator. Incubation was carried out for 24 h to determine production of NO, or lysosomal enzyme activity.

Measurement of NO Release Activity

Nitrite accumulation was used as an indication of NO production, determined by the Griess reaction [6]. One-hundred microliter of culture supernatant or sodium nitrite were mixed with an equal volume of Griess reagent [0.1% (w/v) naphthyl ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] in a microplate well. After 5 min at room temperature, the optical density at 550 nm was measured by using a microplate reader.

Determination of Macrophage Lysosomal Enzyme Activity

Lysosomal enzyme activity was assayed using 96-well flat-bottomed tissue culture plates [29]. Macrophage monolayers in microplates (2×10^5 cells/well) were solubilized by the addition of 25 µl of 0.1% Triton X-100. One-hundred-fifty microliter of 10 mM *p*-nitrophenyl phosphate solution was added as a substrate for acid phosphatase. Then, 50 µl of citrate buffer was added to the well. After incubation for 1 h at 37°C, 25 µl of 0.2 M borate buffer (pH 9.8) was added to the reaction mixture, and the optical density at 405 nm was measured.

Preparation of Spleen Cells

Mice were killed by cervical dislocation. Spleens were freshly removed and were gently teased, and placed in cold RPMI-1640 media. Then, spleen cells were extracted by using a 5-ml syringe. The single cell suspensions were washed three times in cold RPMI-1640 media, counted in 0.2% trypan blue, and adjusted to the desired cell concentration.

One-Way Mixed Lymphocyte Cultures

One-way lymphocyte culture was prepared by a modified method [20]. Spleen cells from BABL/c mice at a density of 2×10^7 cells/ml were incubated at 37°C for 30 min with 25 mg/ml of mitomycin C. The cells were washed twice in cold HBSS containing 10% fetal bovine serum (FBS), incubated again for 10 min at 37°C, and washed before being adjusted to a concentration of $6-8\times10^7$ cells/ml. These mitomycin C-treated cells served as 'stimulator' cells. The second set of spleen cells was prepared from C57BL/6 mice and adjusted to a concentration of 4×10^7 cells/ml to serve as 'responder' cells. One-way mixed lymphocyte cultures were prepared with 2×10^6 stimulator cells plus 2×10^6 responder cells in 200 µl of RPMI-1640 media with or without addition of exopolymers, and in the absence or presence of IFN-γ (20 U/ml) or LPS (the same concentration as exopolymers) in 96-well flat-bottom culture plates. Three replicates of each cell type or combination were made. Each of the plates was incubated in 5% CO_2 at 37°C for 72 h. Methylthiazoletetrazolium (MTT) was added 4 h before termination of culture. At the end of incubation, the plates were centrifuged at 2,500 ×g for 20 min in order to precipitate insoluble formazan. After discarding the supernatant, 0.1 ml of DMSO were added to each well to solublize formazan, and then the optical density at 540 nm was measured by ELISA reader.

Chemical Analysis of Exopolymer

Total protein content of the exopolymer was determined by the method of Lowry *et al.* [18] with bovine serum albumin (BSA) as a standard. The amino acid composition was analyzed by a Biochrom 20 (Pharmacia Biotech. Ltd., U.S.A.) amino acid autoanalyzer with a Na-form column after hydrolysis of the protein. The total sugar and uronic acid contents were determined by the phenol sulfuric acid method [4] and the *m*-hydroxydiphenyl method [1], using a mixture of glucose and galactose (1:1) and galacturonic acid as the respective standards. The sugar composition was analyzed by GC 3600 gas chromatography based on the hydrolysis and acethylation method [9].

Determination of Molecular Weight by HPLC

Molecular weight of the exopolymer was determined by HPLC, using the Shodex GS520, GS320, and GS220 packed column. Standard pullulans (P1600, 800, 400, 200, 100, 50, 20, 10, and 5) were used for the determination of molecular weight as the standard.

RESULTS AND DISCUSSION

Anticomplementary Activity

The yield of water-soluble exopolymer produced from submerged mycelial culture of *P. pini* was 737 mg dry weight/l culture broth. The anticomplementary activity of the exopolymer tended to increase in accordance with an increase in concentration level (Fig. 1). Activity of the exopolymer was higher than the LPS used as the positive control. This exopolymer showed the highest activity at a concentration of 1,000 µg/ml and attained the $ITCH_{50}$ value of 73.2%. However, the concentration above 1,000 µg/ml could not be measured due to the value being out of test range. It has been reported that a polymer from mushroom is closely related with antitumor actions by activating the complementary system [21, 30]. These results suggest that the exopolymer from *P. pini* could have a potential as an antitumor substance.

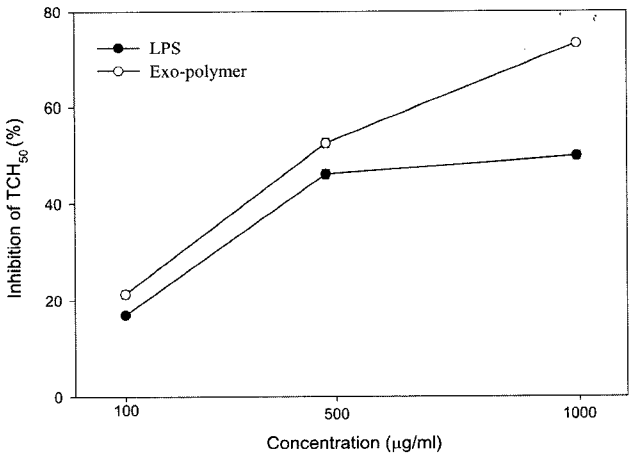

Fig. 1. Anticomplementary activity of the exopolymer produced from the submerged mycelial culture of *Phellinus pini* in various concentrations.

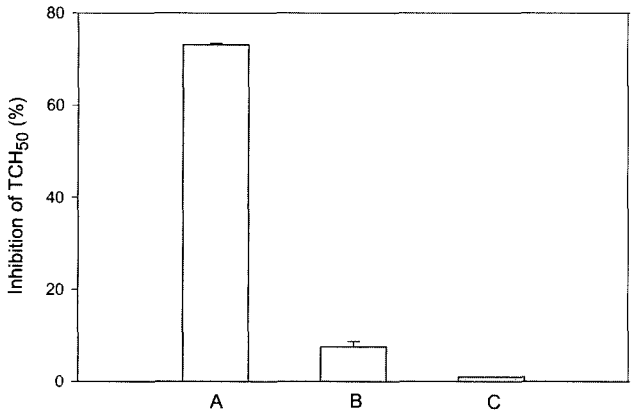

Fig. 2. Anticomplementary activity of the exopolymer produced from the submerged mycelial culture of *Phellinus pini* in the presence or absence of Ca^{++} and Mg^{++}.
A: GVB^{++}, presence of Ca^{++} and Mg^{++}; B: Mg^{++}-EGTA-GVB^{--}, presence of Mg^{++} only; C: EDTA-GVB^{--}, absence of Ca^{++} and Mg^{++}.

Activation Mode of the Anticomplementary Exopolymer

It is known that both Mg^{++} and Ca^{++} ions are needed for the activation of the classical pathway, but only Mg^{++} ion is needed for the activation of the alternative pathway [15]. Under GVB^{++} condition, the anticomplementary activity detected was about 73%, which means active for both classical and alternative pathways, but 7.5% activity, which is active for only the alternative pathway, was recorded in Ca^{++}-depleted experimental condition (Fig. 2). These results demonstrate that the exopolymer mediated *via* both the classical and alternative pathways, where the major pathway was the classical one.

To confirm the above result, crossed immunoelectrophoresis was performed. Two precipitin lines were formed in both GVB^{++} and Mg^{++}-EGTA-GVB^{--} systems (Fig. 3), and the height of the C3a plus C3b precipitin line in GVB^{++} (Fig. 3A) was higher than in Mg^{++}-EGTA-GVB^{--} (Fig. 3B), indicating that the mode of complement activation by exopolymer was activated *via* not only the classical pathway but also the alternative pathway. This pattern of complement activation by the exopolymer was similar to the polysaccharides from AR-arabinogalactan of *Angelica autiloba* [32] and IR-polysaccharide of *Lithospermum enchromum* [33].

Table 1. Neutral sugar and amino acid composition of the exopolymer produced from the submerged mycelial culture of *Phellinus pini.*

Neutral sugar	Composition (molar ratio)	Amino acid	Composition (%)
Arabinose	1.61	Serine	7.43
Xylose	1.00	Asparagine	14.33
Mannose	0.40	Glutamine	21.89
Galactose	0.40	Glycine	9.15
Glucose	30.11	Phenylalanine	4.81
		Threonine	4.13
		Alanine	3.81
		Valine	6.13
		Leucine	3.90
		Lysine	6.90
		Cysteine	2.85
		Isoleucine	3.48
		Methionine	4.02
		Tyrosine	1.92
		Arginine	2.88
		Histidine	1.92
		Proline	0.45
Total sugar content	89.74%	Total protein content	10.26%

Effect of Exopolymer on the Production of Nitric Oxide

The stimulation of murine macrophage by the exopolymer resulted in the expression of an inducible NO synthase (iNOS), which catalyzes the production of a large amount of NO from L-arginine and molecular oxygen [8]. In the present study, the basal level of nitrite in unstimulated peritoneal macrophage was 0.8 μmol/ml. After stimulation with the exopolymer at 25, 50, and 100 μg/ml with 20 U/

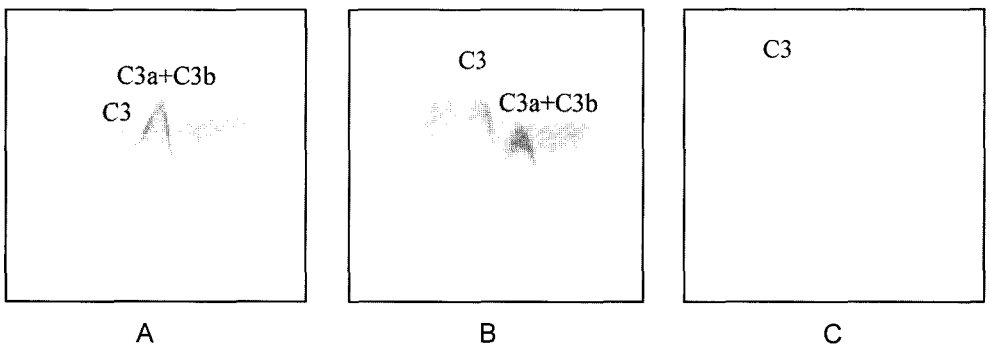

Fig. 3. Crossed immunoelectrophoretic patterns of C3 converted by the exopolymer produced from the submerged mycelial culture of *Phellinu pini* in the presence or absence of Ca^{++} and Mg^{++}.
A: GVB^{++}, presence of Ca^{++} and Mg^{++}; B: Mg^{++}-EGTA-GVB^{--}, presence of Mg^{++} only; C: EDTA-GVB^{--}, absence of Ca^{++} and Mg^{++}.

Table 2. Production of nitric oxide (NO) from thioglycollate elicited peritoneal macrophages of C57BL/6 mice treated with the exopolymer produced from the submerged mycelial culture of *Phellinus pini.*

Treatment	Nitric oxide (Mean±SD) (μM)		
	Control	LPS	Exo-polymer
NC	0.86±0.25		
IFN-γ	11.21±0.42		
25 μg/ml (with IFN-γ)		25.56±1.08	13.15±0.50
50 μg/ml (with IFN-γ)		26.27±0.92	14.39±0.42
100 μg/ml (with IFN-γ)		28.74±2.58	18.21±1.66
100 μg/ml (without IFN-γ)		0.27±0.08	0.14±0.04

NC: negative control (without treatment).

ml of IFN-γ, nitrite synthesis in the macrophage increased to the concentration levels of 13, 14, 18 μmol/ml, respectively. Therefore, this effect was dose-dependent with the improvement of intracellular killing of parasites by macrophage. The exopolymer was not able to activate macrophage to release NO in IFN-γ depleted condition, however, it showed potent NO releasing activity in the presence of IFN-γ (Table 2). It can be stated that the exopolymer stimulates IFN-γ action to increase macrophage activity. During the screening step, we could confirm that optimum concentration of INF-γ for NO synthesis was 20 U/ml (data not shown). These results suggest that the exopolymer could provide a second signal for synergistic induction of NO synthesis in macrophage. However, the NO-producing effect of the exopolymer could not exceed that of LPS at the same concentration levels. Coren [3] demonstrated that stimulation of a murine macrophage with LPS and IFN-γ resulted in high-level NO production. The above results are similar to the effect of herbal plant extracts on the production of NO in mouse macrophages [35].

Effect of Exopolymer on Lysosomal Enzyme Activity

The selective release of lysosomal acid phosphatases (lysosomal enzyme) by mononuclear phagocytes occurs in response to numerous exogenous stimuli [22]. The influence of the exopolymer from *P. pini* on macrophage lysosomal enzyme activity was studied at various concentrations (Fig. 4). When stimulated with the exopolymer at the concentrations of 50, 100, 500, and 1,000 μg/ml, the relative enzyme activity increased by 267, 290, 344, and 336%, respectively, when the activity of negative control (physiological saline) was assumed as 100%. The maximum activity was achieved at the concentration of 500 μg/ml. It is suggested that active fraction of *P. pini* has macrophage activating capacity to lyse foreign substances engulfed by phagocytosis, and the high concentration of active fraction preferably harms macrophage cell by toxicity action. This is similar to the effect of *Agaricus bisporus* fruiting bodies

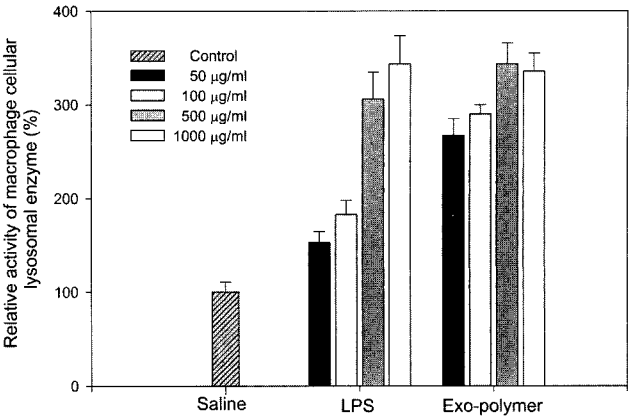

Fig. 4. Macrophage lysosomal enzyme activity of the exopolymer produced from the submerged mycelial culture of *Phellinus pini.*
Saline: negative control.

extract on the production of cellular lysosomal enzyme in mouse macrophage [12]. It has also been reported that the polysaccharides from the fruiting bodies of *Armillariella tabescens* had an antitumor activity on sarcoma 180 in relation to the lysosomal enzyme activation in macrophage [31].

Effect of Exopolymer on the Splenocyte Proliferation Activity

Immune responses of splenocyte responding to alloantigens were determined by MLR. When the activity of the exopolymer was compared with other lymphocyte mitogen (LPS), the ability of splenocyte proliferation was lower than that of LPS at the concentrations of 100 and 200 μg/ml. However, the activity of the exopolymer was higher than LPS at 400 μg/ml. As shown in Fig 5, optimum

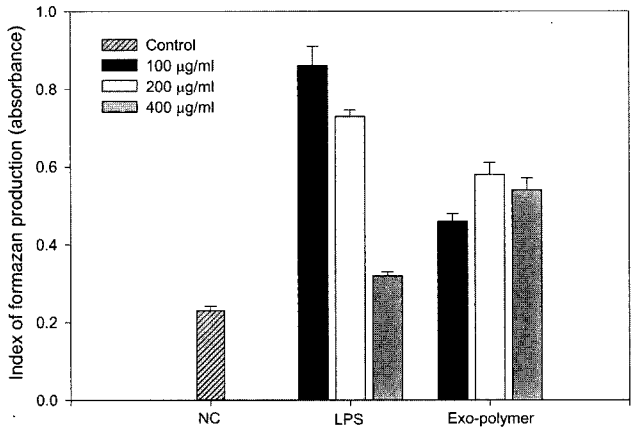

Fig. 5. Effect of the exopolymer produced from the submerged mycelial culture of *Phellinus pini* on splenocyte proliferation by the mixed lymphocyte reaction.
NC: negative control (without treatment); LPS: positive control.

concentration of the exopolymer was 200 μg/ml for the maximum splenocyte proliferation, and it was also increased by 2.6-fold as compared to the control. Moreover, this effect of the exopolymer was similar to splenocytes from C57BL/6 and BABL/c mice, indicating a lack of limitation by mouse genotype (data not shown). Many polysaccharides isolated from *Phellinus* species have been reported to have numerous immunostimulating effects, such as antitumor effect [19], B-lymphocyte stimulating effect [28], and inhibitory effect of tumor growth and metastasis [10]. The roles of the exopolymer from submerged mycelial culture of *P. pini* in splenocyte proliferation have not been explained in detail, however, the exopolymer is thought to have proliferating function due to its higher activity over the NC group. The chemical composition analysis may help understand its biological activity.

The complement system, macrophage, and lymphocyte constitute major host defense systems, acting against invading pathogens. The results demonstrate that the exopolymer produced from a submerged culture of *P. pini* may have three major immune systems.

Chemical Analysis of Exopolymer

The exopolymer obtained from the mycelial culture broth appeared as a single peak when subjected to Sepharose CL-6B column chromatography, and the molecular weight of the exopolymer was estimated to be about 5 kDa by HPLC (Fig. 6).

This polymer had a low molecular weight compared to other water-soluble anticomplementary polysaccharides obtained from the fruiting bodies of mushrooms, which generally ranged in size from 10 kDa to 1,000 kDa [15]. Total protein content of the exopolymer from *P. pini* was 10.26%, and this protein was composed of glutamic acid (21.89%), aspartic acid (14.33%), glycine (9.15%), serine (7.43%), lysine (6.90%), and valine (6.15%) as the major amino acids (Table 1). This was similar to the hot water extract produced from mycelium of *Phellinus linteus* [26]. Total sugar contents of the exopolymer of *P. pini* were 89.74%, and 6.90% of the total sugar was uronic acid. The neutral sugar was mainly composed of glucose (Table 1). The immunomodulatory polymers isolated from mushrooms were reported to contain mainly glucose with β(1→3) and α(1→6) glucosidic linkages [5], and most of the anticomplementary polysaccharides isolated from fungi contain a large amount of glucose [13]. These glucans have been confirmed to exert a variety of effects on immunity, including activation of macrophages, natural killer cells, and neutrophils *in vitro*, and the promotion of resistance against infection and tumors *in vivo* [16].

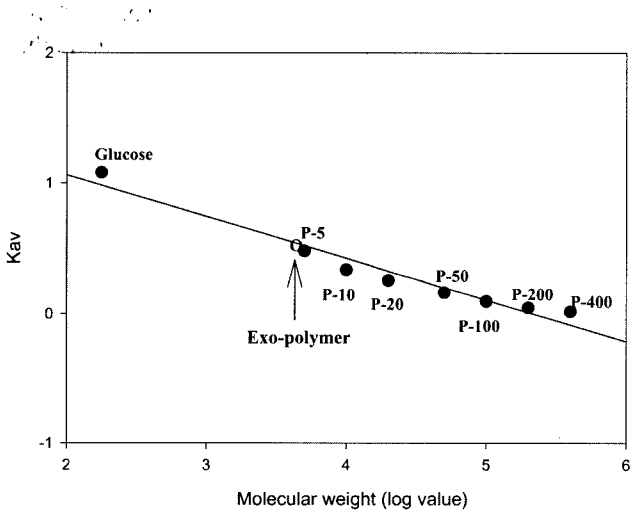

Fig. 6. Determination of molecular weight of the exopolymer produced from the submerged mycelial culture of *Phellinus pini* by HPLC.

P-1600, P-800, P-400, P-200, P-100, P-50, P-20, P-10, and P-5 are standard pullulans of 1600, 800, 400, 200, 100, 50, 20, 10, and 5 kDa, respectively. Kav=(Ve-Vo)/(Vt-Vo) (Vo: Void volume; Vt: Total volume; Ve: Elution volume).

Acknowledgment

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