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Immunomodulating Activities of Water-Soluble Exopolysaccharides Obtained from Submerged Culture of *Lentinus lepideus*

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Immunomodulating activities of water-soluble exopolysaccharides (LL-EX) obtained from submerged mycelial culture of *Lentinus lepideus* were studied and their effectiveness was compared with lipopolysaccharide (LPS). The influence of the LL-EX on macrophage cellular lysosomal enzyme activity was to stimulate up to 267%, 392%, and 464% at the level of 10, 50, and 100 μg/ml, respectively. When the LL-EX was further fractionated into LL-Fr.I and Fr.II by Sepharose CL-6B gel chromatography, the cellular lysosomal enzyme activity of LL-Fr.II (2.1-fold) was higher than Fr.I (1.2-fold). Moreover, both LL-Fr.I and Fr.II stimulated the cytokines IL-1β, TNF-α, and IL-6 in macrophages. In mixed lymphocyte reaction, LL-Fr.I and Fr.II enhanced the splenocyte proliferation up to 1.2-fold and 1.4-fold (50 μg/ml), respectively, stimulating only T lymphocytes. The fractions of LL-EX not show any direct toxicity against human gastric adenocarcinoma cell (AGS). The molecular masses of LL-Fr.I and Fr.II were estimated to be about 1,986 kDa and 21 kDa, respectively. The total sugar and protein contents of the two fractions were 84.97% and 69.88%, and 15.03% and 30.12%, respectively. The sugar and amino acid compositions of the LL-Fr.I and Fr.II were also analyzed in detail.

Keywords: *Lentinus lepideus*, immunomodulating, IL-1β, IL-6, TNF-α, T lymphocyte

The search for novel polysaccharides with antitumor properties stems from the basic shortcomings of cancer chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells. Hence, the discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical sciences [23]. Thus, numerous studies conducted on antitumor activity possessed by natural compounds, including compounds of mushroom origin, have contributed to the search of possibilities to resolve this global problem [4, 6, 20, 40].

Mushroom polysaccharides exert their antitumor action mostly *via* activation of the immune response of the host organism. Indeed, many polysaccharides have been reported to exhibit anti-inflammatory [10], hypotensive [16], antibiotic [19], antiviral [33], and hypoglycemic [44] activities. These substances are regarded as biological response modifiers [40]. This means that it causes no harm and places no additional stress on the body; it helps the body to adapt in various environmental and biological stresses; and it exerts a nonspecific action on the body, supporting some of the major systems, including nervous, hormonal and immune systems, as well as regulatory functions [2]. Polysaccharides prepared from many mushrooms including *Lentinus edodes* [3], *Agaricus blazei* [22], and *Grifola frondosa* [20] suppress tumor growth *in vivo* by controlling the immune system in the host [48]. In addition, cytokines have been observed to be regulated by various mushrooms. For example, lentinan, a polysaccharide isolated from *L. edodes*, induces a variety of cytokines, including TNF-α, IL-2, IL-1α, IL-1β, IFN-γ, and M-CSF [24, 25].

L. lepideus is an edible mushroom found around the world. The endopolysaccharide extracted from the fruiting body of *L. lepideus* increases the expression of various important cytokines that play key roles in the turning on the innate immune response [5]. However, the exopolysaccharide obtained from submerged culture of *L. lepideus* has not been reported to have immunomodulating and antitumor effects. Therefore, in the present investigation, the immunomodulating activities of exopolysaccharide obtained from *L. lepideus* were studied and the effort was made to purify and fractionate active compounds from the

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crude extract in order to obtain the pure component and chemical compositions.

Materials and Methods

Strain and Culture Medium

L. lepideus, obtained from the Korean Agriculture Culture Collection in South Korea, was grown in potato dextrose broth on a rotary shaker (120 rpm) at 25°C. After 7 days, 100 ml of culture broth was aseptically homogenized and inoculated at 2% (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 4.2 before sterilization.

AGS was obtained from Korean Cell Line Bank of Seoul National University. The cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml of penicillin, and 100 μl/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2, and the medium was changed every 2 days. Cells were split in a 1:3 ratio at every other day by tripsinization.

Preparation and Purification of Water-Soluble Exopolysaccharide

Culture broth was harvested by centrifugation (10,447 ×*g*, 20 min) and the supernatant was mixed with an 80% final concentration of ethanol. Ethanol precipitate was collected, dialyzed against distilled water, and lyophilized to obtain an LL-EX. The preparation and isolation details are shown in Fig. 1. The LL-EX solution was applied to a column (6.6×50 cm) of Sepharose CL-6B, which had been equilibrated with 0.2 M NaCl, and was eluted with the same solution at 4°C.

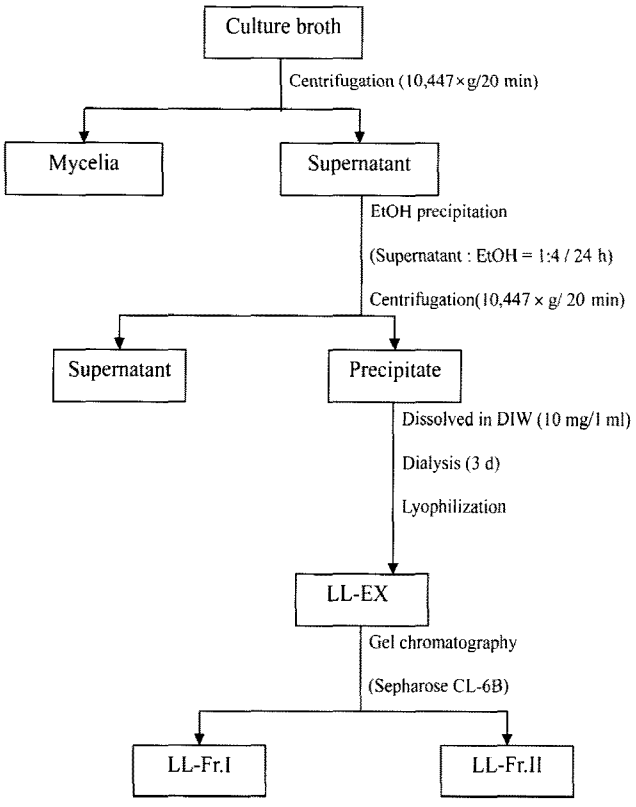

Fig. 1. Schematic diagram depicting the process used to recover exopolysaccharide from a culture broth of *Lentinus lepideus*.

Experimental Animals and Breeding Conditions

Male 6-week-old mice (C57BL/6 and BALB/c strains) weighing approximately 25 g were purchased from Daehan Biolink, and housed in plastic cages. The mice were housed at constant temperature (22±2°C) and humidity (55±5%) under a 12-h light/dark cycle. The mice were fed a commercial pellet diet (Sam Yang Co., Korea) throughout the experimental period.

Preparation of Mouse Macrophages

Macrophages were harvested from the mice three days after an intraperitoneal (i.p.) injection of 3 ml of 10% thioglycolate medium. Cell density was adjusted to 2×10^6 cells/ml with Dulbecco's modified Eagles medium (DMEM) buffer supplemented with 10% FBS. Thereafter, each well of a 96-well microplate was inoculated with 200 μl of the cell suspension (2×10^6 cells/well). Adherent macrophages were isolated by incubating the cells for 2 h in CO_2, and this was followed by vigorously shaking and washing the plate to remove nonadherent cells. Cultures were incubated with or without test exopolysaccharide additions, and in the absence or presence of LPS, at 37°C in a 5% CO_2 humidified incubator.

Determination of Macrophage Cellular Lysosomal Enzyme Activity

Lysosomal enzyme activity was assayed using 96-well flat bottomed tissue culture plates [37]. Macrophage monolayers in the microplates (2×10^6 cells/well) were solubilized by adding 25 μl of 0.1% Triton X-100. *p*-Nitrophenyl phosphate (*p*NPP) solution (150 μl; 10 mM) was then added per well as a substrate for acid phosphatase, and this was followed by 50 μl of citrate buffer per well. After incubating for 1 h at 37°C, 25 μl of 0.2 M borate buffer (pH 9.8) was added to each well, and optical densities were measured at 405 nm.

Determination of Cytokines

The levels of cytokines IL-1β, IL-6, and TNF-α in the culture supernatants were assayed using an enzyme-linked immunosorbent assay (ELISA) [32]. Mouse IL-1β, IL-6, and TNF-α ELISA kits were purchased from eBioscience (San Diego, U.S.A.). In brief, 96-well microplates were coated overnight at 4°C with 100 ml of capture antibody (4 μg/ml) diluted in 0.1 M coating buffer. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 μl of 10% FBS in PBS for 2 h at room temperature. After washing, triplicate supernatant macrophage culture samples of 50 μl were added to each well. After 18 h of incubation at 4°C, the wells were washed and incubated with 100 μl (2 μg/ml) of the biotinylated monoclonal antibodies anti-IL-1β, anti-IL-6, and anti-TNF-α for 45 min at room temperature. After a final wash, the substrate was added and the stop solution was added to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was measured from a standard curve established with the appropriate recombinant cytokine.

Preparation of Spleen Cells

Mice were killed by cervical dislocation, and spleens were removed immediately and placed in cold RPMI-1640 medium. Spleen cells were extracted and separated using a 5-ml syringe. Cell suspensions were washed three times in cold RPMI-1640 medium, counted in

0.2% trypan blue, and adjusted to concentration. T and B lymphocytes were separated using the nylon wool enrichment method [8].

Mixed Lymphocyte Culture (MLC)

One-way lymphocyte cultures were prepared using a modification of the method described by Murgita and Tamasi [29]. Spleen cells from BALB/c mice at a density of 2×10^7 cells/ml were incubated with mitomycin C (25 μg/ml) at 37°C for 30 min. The cells were washed twice with cold Hank's balanced salt solution (HBSS) containing 10% FBS, reincubated for 10 min at 37°C, and washed before being adjusted to a concentration of $6–8\times10^7$ cells/ml. Mitomycin C-treated cells served as "stimulator" cells. A second set of spleen cells were 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 in 96-well microplates. Three replicates of each cell type or combination were made. Plates were then incubated in 5% CO_2 at 37°C for 72 h. Methylthiazoletetrazolium (MTT) was added 4 h prior to the termination of culture. After this incubation, plates were centrifuged at 3,000 rpm for 20 min in order to precipitate insoluble formazan. After discarding the supernatant, 0.1 ml of dimethyl sulfoxide (DMSO) were added to each well to solubilize the formazan, and optical densities were measured at 540 nm using an ELISA reader.

Determination of Direct Cytotoxicity

Direct cytotoxicity was assessed by the MTT assay based on the reduction of MTT into formazan dye by active mitochondria. Briefly, the cells were placed in 96-well microplates at a density of 5×10^4 cells/well in RPMI-1640 culture medium containing 10% FBS at 37°C, 5% CO_2. After 28 h, the cells were washed and placed in culture medium with different concentrations of LL-Fr.I and Fr.II for 48 h. Thereafter, 20 μl of MTT solution (5 mg MTT/ml in PBS) was added to each well of a 96-well microplate and incubated for 4 h. After washing, the formazan dye precipitates, the amount of which is proportional to the number of live cells, were dissolved in 100 μl of DMSO. The absorbance was measured at 540 nm using an ELISA. The inhibition rate of cell growth was calculated by the following formula: mean value of (control group-treated group)/ control group ×100%.

Determination of Molecular Weight

Molecular weights of LL-Fr.I and Fr.II were determined by Sepharose CL-6B gel column chromatography [43]. Standard dextran series T-2000 (2×10^6), T-500 (5×10^5), T-70 (7×10^4), T-40 (4×10^4), T-10 (1×10^4), and glucose (180) were used as the standard molecules, and the calcultions were done as follows:

$$Kav=(V_e-V_o)/(V_t-V_o).$$

Kav: partition constant

(V_o) void volume, (V_t) total volume, (V_e) elution volume.

Analysis of Protein and Sugar Compositions

Total protein contents of the LL-Fr.I and Fr.II were determined by the method of Lowry *et al.* [26] with bovine serum albumin (BSA) as a standard. The protein was hydrolyzed and the amino acid composition was analyzed by a Biochrom 20 (Pharmacia Biotech. Ltd., U.S.A.) amino acid autoanalyzer with a Na-form column. The total sugar content was determined by the phenol sulfuric acid method using a glucose and arabinose mixture (1:1) as a standard [7]. The sugar composition was analyzed by a VarianGC3600 gas chromatography equipped with a flame-ionization detector on a SP-2380 capillary column (15 m×0.25 mm i.d., 0.2-μm film: Supelco) based on the hydrolysis and acetylation method [15].

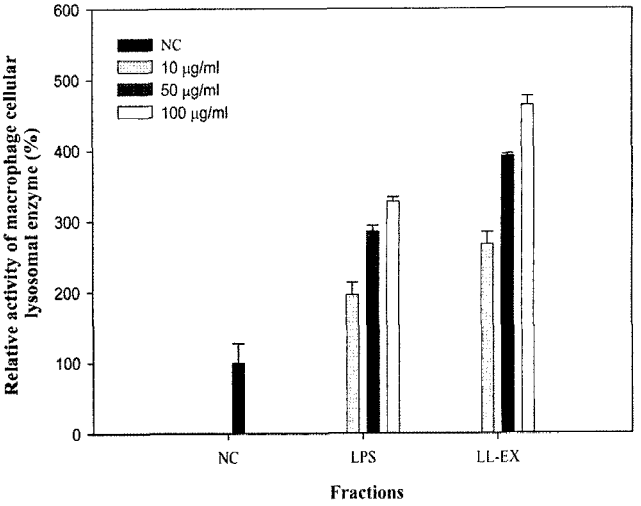

Fig. 2. Macrophage cellular lysosomal enzyme activities of exopolysaccharides obtained from *Lentinus lepideus*.

NC: Saline was used for the negative control; LPS: Lipopolysaccharide was used for the positive control. Concentration of macrophage was 2×10^6 cells/ml. Each value is the mean±SD for triple determinations.

RESULTS AND DISCUSSION

Macrophage Lysosomal Enzyme Activities of LL-EX

Lysosomal enzyme and phagocytic activities are crucial aspects of macrophage functional assessments [39]. The

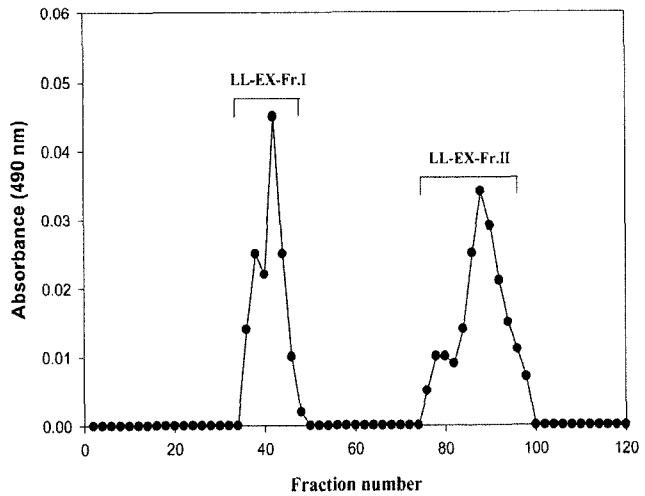

Fig. 3. Gel chromatography pattern of the LL-EX produced from submerged culture of *Lentinus lepideus* on the Sepharose CL-6B column.

LL-EX was dissolved in 0.2 M NaCl solution. The volume of each fraction was 5 ml, and the eluates were checked by measuring the absorbance.

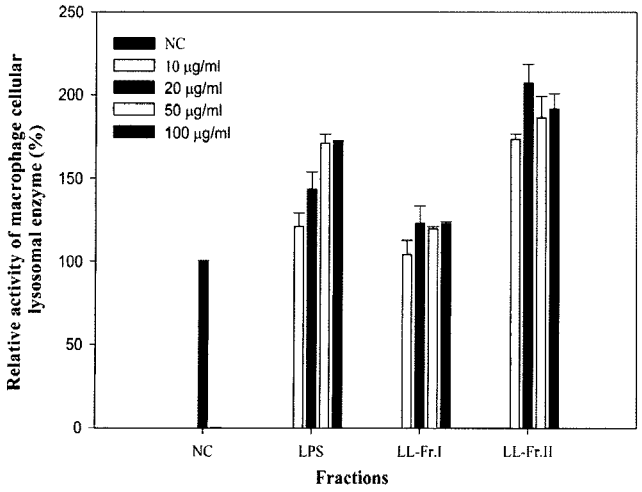

Fig. 4. Macrophage cellular lysosomal enzyme activities of LL-EX fractions obtained from submerged culture of *Lentinus lepideus* at various concentrations.
NC: Saline was used for the negative control; LPS: Lipopolysaccharide was used for the positive control. Concentration of macrophage was 2×10^6 cells/ml. Each value is the mean±SD for triple determinations.

production of lysosomal acid phosphatases (lysosomal enzymes) by mononuclear phagocytes occurs in response to numerous exogenous stimuli [13]. The influence of the LL-EX obtained from submerged mycelial culture of *L. lepideus* on macrophage cellular lysosomal enzyme activity was measured at various concentrations (Fig. 2). The LL-EX stimulated the relative enzyme activity up to 267%, 392%, and 464% at the levels of 10, 50, and 100 μg/ml, respectively. When the activities of LL-Fr.I and Fr.II were compared, the LL-Fr.II was higher than LL-Fr.I (Fig. 3, 4). The LL-Fr.II showed higher activity at all concentrations, compared with the positive control (LPS). These results suggested that LL-Fr.II and Fr.I have macrophage activating capacity to lyse foreign substances engulfed by phagocytosis. Lysosomal enzyme acid phosphatase is an important part of the macrophage arsenal, which is the first step of the macrophage response against invading microorganisms. The elevated lysosomal enzyme activity facilitates a more efficient eradication of foreign substances [31].

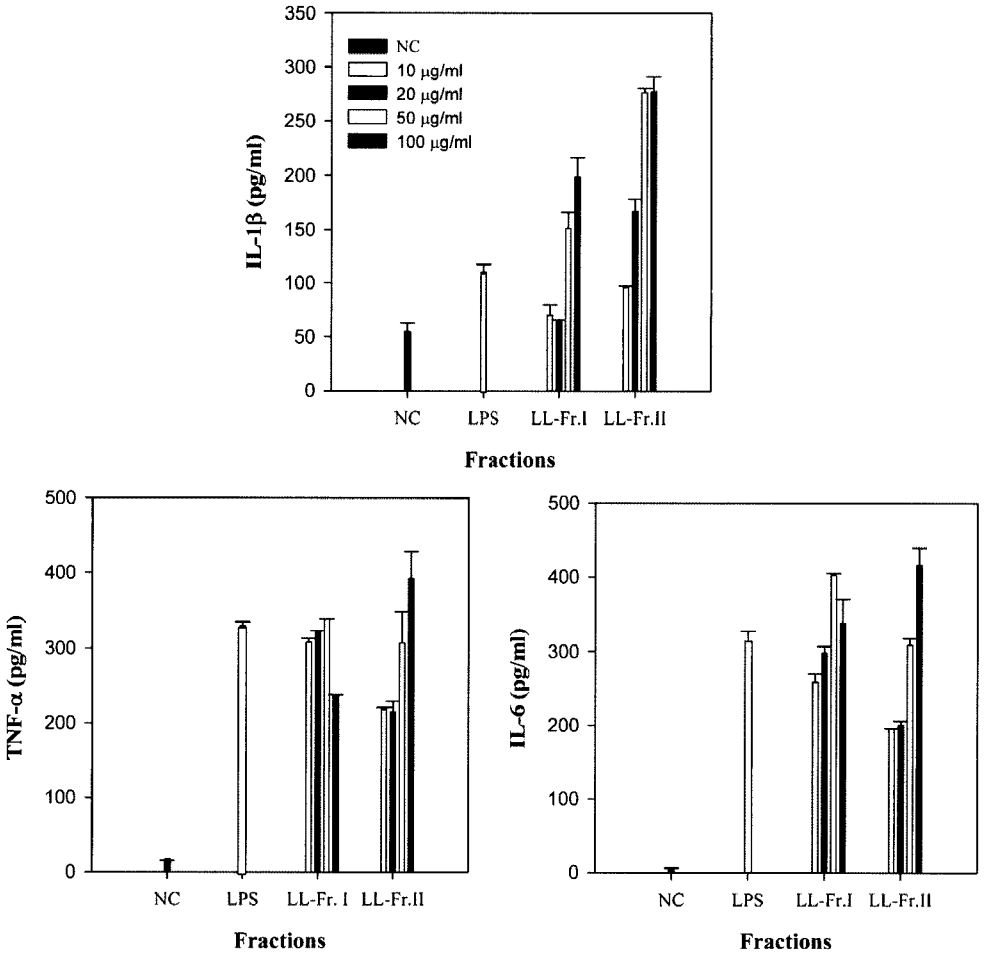

Fig. 5. Effects of LL-EX fractions LL-Fr.I and Fr.II obtained from submerged culture of *Lentinuse lepideus*, at various concentrations on the production of cytokines in macrophages.
NC: Saline was used for the negative control; LPS: Lipopolysaccharide was used for the positive control. Concentration of macrophage was 2×10^6 cells/ml. Each value is the mean±SD for triple determinations.

Effects of LL-Fr.I and Fr.II on the Production of Cytokines

Cytokines are known as biologic response modifiers that modulate inflammation, immunity, and hematopoiesis. Hence, polysaccharides also known as a kind of cytokines [11, 28]. The cytokines (IL-1β, TNF-α, and IL-6) production secreted by activated macrophage supernatant is shown in Fig. 5. We found that LL-Fr.I and Fr.II at 100 μg/ml increased the levels of IL-1β to 1.8-fold and 2.7-fold, respectively, compared with the LPS. The maximum levels of TNF-α activation by the LL-Fr.I and Fr.II were observed at 50 μg/ml and 100 μg/ml, respectively. The highest levels of IL-6 were also achieved by LL-Fr.I at 50 μg/ml and Fr.II at 100 μg/ml (Fig. 5). The production of cytokines by LL-Fr.II was dose dependent but not for LL-Fr.I. However, these results suggested that both LL-Fr.I and Fr.II could induce production of the inflammatory effector cytokines IL-1β, TNF-α, and IL-6 in macrophages. The production of cytokines by activated macrophages is central to their immunoregulatory role and in the orchestration of a robust immune response in macrophages, which maintain an effective immune response at the site of inflammation and malignancy [38].

Effects of LL-Fr.I and Fr.II on Splenocyte Proliferation Activity

It has previously been reported that several carbohydrate compounds isolated from fungi contain mitogenic activity for murine lymphocytes [35]. To test whether LL-Fr.I and Fr.II also have a similar mitogenic effect, we examined their effects on the proliferation of murine splenocytes by MLC. When the activities of the LL-Fr.I and Fr.II were compared with other lymphocyte mitogen (LPS), the ability of splenocyte proliferation with LPS was found to be similar with LL-Fr.I at 20 μg/ml and Fr.II at 50 and 100 μg/ml in concentration (Fig. 6). We also found that LL-Fr.I (20 μg/ml) and Fr.II (50 μg/ml) enhanced the splenocyte proliferation up to 1.3-fold and 1.4-fold, compared with the control. This response, as well as the subsequent differentiation into effector lymphocyte, involves interactions between lymphocytes and macrophage, both have been shown to mediate various types of cell-mediated immune function. Previous studies indicated that many polysaccharides obtained from mushrooms could stimulate the proliferation of splenocyte [12, 18]. These results suggested that LL-Fr.I and Fr.II are efficient polyclonal activators of splenocytes.

Effects of LL-Fr.I and Fr.II on T and B Lymphocytes Proliferation Activity

The lymphoproliferative potential of the exopolysaccharide fractions was also studied in T and B lymphocytes. As shown in Fig. 7, the activities of LPS- or concanavalin A (Con-A)-treated groups were quite different from those of T and B lymphocytes. The number of T lymphocytes in the Con-A-treated group was increased over that of control group (NC) in a dose-dependent manner. However, the proliferation of T lymphocyte was not changed by LPS. These results indicate that T and B lymphocytes were successfully separated from splenocyte. T lymphocyte proliferations induced by LL-Fr.I increased in a dose dependent manner, compared with the control. Maximum T lymphocyte proliferation by LL-Fr.II was observed at 20 μg/ml, which represented a 1.6-fold enhancement. However, B lymphocyte proliferations induced by LL-Fr.I and Fr.II were little increased at all concentrations. Therefore, LL-

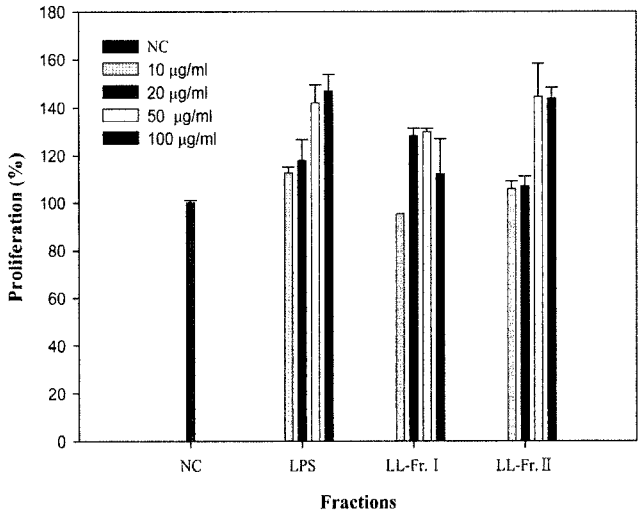

Fig. 6. Effects of LL-EX fractions LL-Fr.I and Fr.II obtained from submerged culture of *Lentinus lepideus*, at various concentrations on splenocyte proliferation by the mixed lymphocyte culture.
NC: Saline was used for the negative control; LPS: Lipopolysaccharide was used for the positive control. Concentration of macrophage was 2×10^6 cells/ml. Each value is the mean±SD for triple determinations.

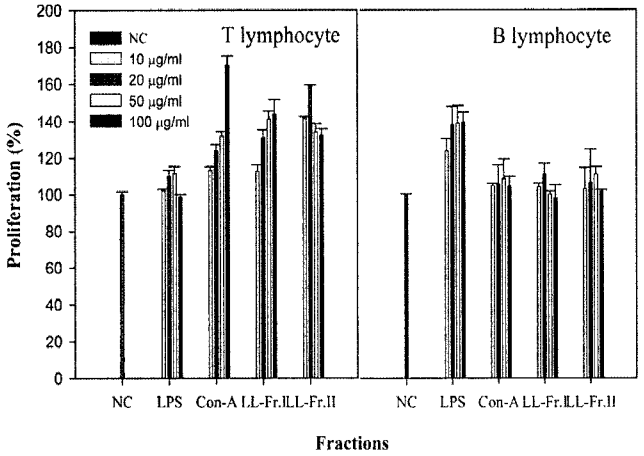

Fig. 7. Effects of LL-EX fractions LL-Fr.I and Fr.II obtained from submerged culture of *Lentinus lepideus*, at various concentrations on T and B lymphocytes proliferation.
NC: Saline was used for the negative control; LPS: Lipopolysaccharide was used for the positive control of B lymphocyte; Con-A: Concanavalin A was used in positive control of T lymphocyte. Concentration of splenocyte was 2×10^6 cells/ml. Each value is the mean±SD for triple determinations.

Fr.I and Fr.II have a mitogenic effect on T lymphocyte, but not on B lymphocyte. It has been reported that the water-soluble glycan obtained from the endopolysaccharide of *L. lepideus* induced B lymphocyte [14]. It could be due to structural and compositional differences between endo- and exopolysaccharides. Polysaccharides represent a structurally diversified class of macromolecules, and this structural variability can profoundly affect their cell-type specificities, especially with respect to T and B lymphocytes. For example, β (1→3)-glucans isolated from *L. edodes* and *Schizophyllum commune* were found to stimulate T lymphocyte but not B lymphocyte [1, 27]. Whereas polysaccharides from *A. bisporus* activated both B and T lymphocytes [17], and other polysaccharides from *Angelica gigas* Nacki were found to activate only B lymphocyte [8]. Our results suggested that the major target of the both fraction were the T lymphocytes.

Effects of LL-Fr.I and Fr.II on Direct Cytotoxicities on AGS

Polysaccharides from mushrooms usually does not attack the cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. It has been verified in many experiments, such as the loss of the antitumor effect of polysaccharides in neonatal thymectomized mice or after administration of antilymphocyte serum [30]. Such results suggest that the antitumor action of polysaccharides requires an intact T cell component and that the activity is mediated through a thymus-dependent immune mechanism. Moreover, the antitumor activity of lentinan from *L. edodes* was inhibited by pretreatment with antimacrophage agents. Thus, the various effects of polysaccharides are thought to be due to a potential of the response of precursor T cells and macrophages to cytokines produced by lymphocytes after specific recognition of tumor cells [40]. As shown in Fig. 8, the direct cytotoxicities of LL-Fr.I and Fr.II were tested with AGS. LL-Fr.I and Fr.II were not toxic. The results of the present study suggest that LL-Fr.I and Fr.II selectively and efficiently activate macrophages, cytokines in macrophages, and T lymphocyte, but not B lymphocyte.

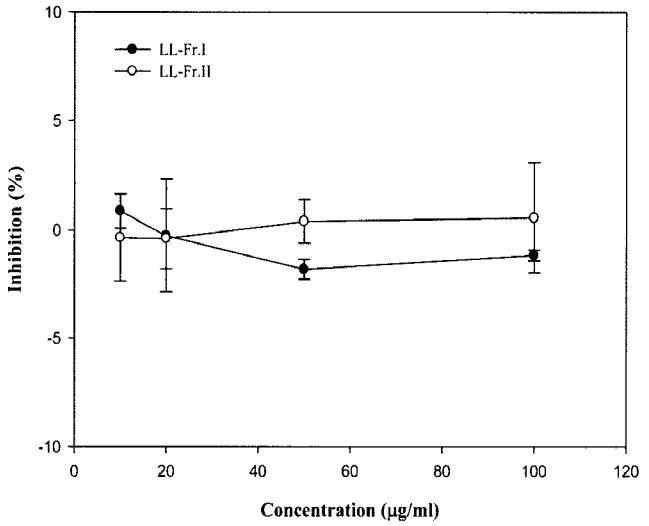

Fig. 8. The direct cytotoxicities of LL-EX fractions LL-Fr.I and Fr.II obtained from submerged culture of *Lentinus lepideus*, at various concentrations on the growth of AGS.
Concentration of AGS was 5×10^4 cells/ml. Each value is the mean±SD for triple determinations.

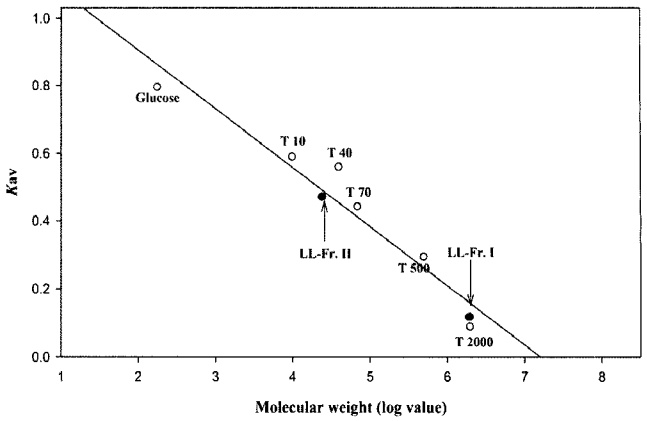

Fig. 9. Determination of the molecular weights of LL-Fr.I and Fr.II by Sepharose CL-6B gel chromatography.
Standards of dextran series : T-70 (7×10^4), T-40 (4×10^4), T-10 (1×10^4), and glucose (180). $K_{av}=(V_e-V_o)/(V_t-V_o)$ (V_o, void volume; V_t, total volume; V_e, elution volume).

Chemical Analysis

The molecular masses of the two fractions isolated from *L. lepideus* exopolysaccharides are shown in Fig. 9. The molecular masses of LL-Fr.I and Fr.II were estimated to be about 1,986 kDa and 21 kDa, respectively. As observed in the study of Jeong *et al.* [12] working with *Phellinus pini*, exopolymers having low molecular mass (5 kDa) showed high immunomodulating activity. The present data indicate that the immunomodulating activity highly depends on the molecular weight of the compound concerned, as reported by several investigators [12, 42].

Table 1. Sugar composition of LL-Fr.I and Fr.II on the Sepharose CL-6B gel chromatography.

	LL-Fr.I	LL-Fr.II
Total sugar (%)[a]	84.97	69.88
Neutral sugar (%)[a]		
Fucose	3.87	4.98
Ribose	2.40	1.74
Arabinose	-	-
Xylose	35.18	1.02
Mannose	47.74	50.85
Galactose	1.07	38.76
Glucose	9.74	2.65

[a]Calculated on the basis of total neutral sugar.

Table 2. Amino acids composition of LL-Fr.I and Fr.II on the Sepharose CL-6B gel chromatography.

	LL-Fr.I	LL-Fr.II
Total protein (%)[a]	15.03	30.12
Amino acid (%)[a]		
Aspartic acid	14.46	4.19
Threonine	10.73	4.32
Serine	14.34	6.94
Glutamic acid	12.72	3.48
Proline	-	4.20
Glycine	18.98	-
Alanine	17.60	65.04
Cystine	-	-
Valine	-	2.46
Methionine	-	-
Isoleucine	-	2.02
Leucine	-	2.49
Threonine	-	-
Phenylalanine	11.16	2.28
Histidine	-	-
Lysine	-	1.80
Arginine	-	0.77

[a]Calculated on the basis of total amino acids.

The two fractions, LL-Fr.I and Fr.II, of *L. lepideus* exopolysaccharide were found to contain 84.97% and 69.88% sugar contents, respectively (Table 1). LL-Fr.I contained mainly mannose (47.74 %), xylose (35.18%), and glucose (9.74%), whereas LL-Fr.II had mannose (50.85%) and galactose (38.76%). Most polymers isolated from mushrooms with immune-modulating activities are known to contain a large amount of mannose [12, 23]. These polymers have been reported as stimulating factors of immunity, including activation of macrophages, natural killer cells, and neutrophils [21, 41]. The fractions LL-Fr.I and Fr.II contained mainly mannose, a polysaccharide that might bind to the receptor of macrophages and lead to activation of immunity. Mannose-rich polysaccharides can activate the immune system at the molecular level, and may also collaborate with Toll-like receptors for the activation of immunity [23]. The total protein contents of LL-Fr.I and Fr.II were 15.03% and 30.12%, respectively. The amino acids compositions of these two subfractions are summarized in Table 2. The LL-Fr.I contained mainly glycine (18.98%) and alanine (17.60%), followed by aspartic acid (14.46%) and serine (14.34%), and the LL-Fr.II contained mainly alanine (65.04%). This result was similar to the exopolysaccharide of *Grifola frondosa* [45]. These data represent that the isolated exopolysaccharide fractions could be a glycopeptide or proteoglycan, which are composed of carbohydrate and amino acids. Shiyan and Bovin [36] reported that glycoprotein influenced cytokines production, such as IL-1, IL-2, IL-6, and TNF-α. The results of the present study suggested that the carbohydrate and protein moieties in each fraction contribute to the expression of the activity.

Acknowledgment

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