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RESEARCH ARTICLE

Antitumor and Immunomodulating Activities of Endo-biopolymers Obtained from a Submerged Culture of *Pleurotus eryngii*

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Abstract Antitumor effect of endo-biopolymer (EBP) obtained from submerged mycelial culture of *Pleurotus eryngii* was investigated by using Sarcoma-180 bearing mice. The administration of EBP, intraperitoneally (10 to 80 mg/kg BW) at all dose ranges, inhibited the growth of the solid tumor and simultaneously increased the activity of natural killer (NK) cells significantly. While, 40 mg/kg BW treated group reduced the tumor formation (53.1%), and increased the NK cell activity of splenocytes (25.0%) to a great extent. The complement activity of the EBP was 43.1% (1,000 μg/mL). Phosphatase activity of macrophage was found to be increased up to 35.3% (100 μg/mL) when compared with control. The chemical composition of EBP was found to contain carbohydrate (77.5%) and protein (21.6%). The major component sugars of EBP were glucose and mannose, while glycine, glutamic acid, and aspartic acid mainly constituted the protein moiety. These results revealed that EBP obtained from submerged culture of *P. eryngii* stimulate not only immune system but also antitumor activity.

Keywords: antitumor activity, endo-biopolymer, *Pleurotus eryngii*, Sarcoma-180, immunomodulating activity

Introduction

Edible mushrooms are known to be a flavorful foodstuff having medicinal importance in folk medicine. Recently, much attention has been paid to their therapeutic values, such as antitumor (1), immunomodulatory (2), hypoglycemic (3), and hypolipidemic activities (4). Biologically active substances generated in mushrooms are known to have components such as polysaccharides, terpenoids, polysaccharide-peptide complexes, and proteins (5).

Pleurotus sp. is edible and which also have several biological effects, as they contain important polysaccharides (6). *Pleurotus eryngii* a tetrapolar basidomycete belonging to Pleurotaceae (Agaricales), is distributed widely in areas of southern Europe, central Asia, and North Africa. In Japan, *P. eryngii* has recently become popular as a new edible mushroom because of its good taste and biological functions, so its production has increased rapidly in recent years. Antitumor activity of the crude polysaccharide of *Pleurotus* sp. fruiting body has already been carried out by several workers (7-11).

As the antitumor activity of the biopolymer produced by *P. eryngii* during submerged fermentation has not been reported, hence, a dose-dependent antitumor and immunomodulating effects of endo-biopolymer (EBP) on tumor-bearing mice has been reported in the present communication.

Materials and Methods

Strain and production of EBP The experimental organism (*Pleurotus eryngii*, a mushroom, KACC 50037) obtained from the Korean Agricultural Culture Collection (KACC) was maintained (4°C) on potato dextrose agar (PDA, Difco, Detroit, MI, USA) slant and subcultured in every 3 months. The seed cultures of *P. eryngii* was grown in 250-mL Erlenmeyer flasks containing 100 mL of potato dextrose broth (pH 4.0) and incubated at 25°C/150 rpm for approximately 10 days. The mushroom complete medium

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(MCM) of the following composition (g/L): glucose 20, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, yeast extract 2.0, and peptone 2.0 with pH 4.0 was used for the production of EBP. The submerged fermentation was carried out (25°C/150 rpm) in 500-mL Erlenmeyer flasks containing 200 mL of the media. The EBP was extracted from the mycelial pellet by hot water extraction followed by centrifugation (10,447×g for 20 min) and by treating the supernatant with 4 volumes of ethanol. The EBP thus obtained was filtrated and lyophilized, for further use.

Animal BALB/c male mice (5 weeks of age), approximately 22 g, were purchased from Daehan Biolink Co., Ltd. (Umsong, Chungbuk, Korea), and housed in plastic cages. The animal room was maintained at constant temperature (22±2°C) and humidity (55±5%) with 12 hr cycle of light and dark. The mice were fed commercial pellet diet (Sam Yang Co., Wonju, Gangweon, Korea) throughout the experimental period. The protocol of all animal experiments was approved by the Institutional (Daegu University) Animal Ethics Committee and performed under the guidelines of the Laboratory Animal Experiment Committee of Korea Food Research Institute.

Assay of antitumor activity The Sarcoma-180 tumor cell line was supplied by the Korea Cancer Cell Line Bank (Seoul, Korea). Tumor was induced by injection of sarcoma-180 cell (6.0×10^6 cells/0.2 mL in phosphate buffered saline: PBS) into the left groin of BALB/c male mice. The EBP (10-80 mg/kg BW) prepared in PBS was intraperitoneally injected daily for 28 days at 24 hr after inoculation of tumor cells. The same volume of PBS injected intraperitoneally was served as control. The solid tumor was allowed to grow in the mice for 28 days before it was removed from the mice, and weighted. Moreover, spleens were dissected to measure the NK cell activity. The antitumor activity of the tested samples was expressed as an inhibition ratio (%) calculated as: [(A-B)/A]×100, where A and B are the average tumor weights of the control and treated groups, respectively (12,13).

Measurement of NK cell activity Splenocytes were isolated by a modified method described by Mishell and Shiigi (14). Spleen was dissected aseptically and grounded in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 100 U/mL of penicillin-streptomycin (Gibco). The cell suspension was filtered through 70-μm mesh. The splenocytes were collected by centrifugation and resuspended in the same media. The NK cells were isolated by centrifugation (400×g for 30 min at 18°C) using histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). The NK cells resuspended in the RPMI-1460 with 10% of fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 100 U/mL of penicillin-streptomycin were allowed to adhere in a culture flask (1 hr at 37°C/5% CO_2 atmosphere). Non-adherent NK cells used as the effectors cells were collected by centrifugation and resuspended. Over 90% of cell viability was used in this experiment.

The NK cell activity was estimated using a dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) (15). The YAC-1 murine lymphoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and used as target cell. YAC-1 cells (2.0×10^5 cells/mL) maintained in complete medium were collected by centrifugation and later the cell (5.0×10^4 cells/mL) were resuspended. For the NK cell activity assay, 50 μL of each effectors cells and YAC-1 cells were added to each well containing 96-well flat-bottomed microplate. Each treated splenocyte from mice was assayed in triplicate at the ratio of each effector/target cell (200:1). After 3 days of inoculation at 37°C, the cells loaded with 10 μL of freshly prepared MTT (5 g/mL) were further incubated (37°C for 4 hr). The 25 μL of sodium dodecyl sulfate (SDS, 10% in 0.02 N HCl) was added to each well and the microplate was left for 30 min at room temperature to develop color. The optical density (OD) was measured (540 nm) by enzyme-linked immunosorbent assay (ELISA) reader (Universal microplate reader EL800; Bio-Tek Instruments Inc., Winooski, VT, USA). The percentage cell cytolysis was calculated using the following equation:

Cytolysis %=[1 (OD of non-lysed target cells OD of effector cells)/OD of target cells]×100

Complementary activity assay The complementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement (16). Fifty μL of EBP solution in water was mixed with equal volumes of normal human serum (NHS) and gelatin veronal buffered saline (GVB, pH 7.4) containing 500 μg MG^{++} and 150 μg Ca^{++}. The mixtures were incubated (37°C for 30 min) and the residual total complement hemolysis (TCH_{50}) was determined using IgM hemolysin sensitized sheep erythrocytes (1.0×10^8 cells/mL) (Denka Seiken Co., Ltd., Nigata, Japan). The NHS was incubated with deionized water (DIW) and GVB^{++} to provide a control. The complementary activity of EBP was expressed as the percentage inhibition of the TCH_{50} of control.

Preparation of macrophage Macrophages were harvested from mice 3 days after an intraperioneal injection (3 mL of 10% thioglycolate medium). Cell density (1.0×10^6 cells/mL) was adjusted with Hank's balance salt solution (HBSS) buffer, supplemented with 10% FBS. Later, each

well of a 96-well micro plate was inoculated with 200 mL of the cell suspension (2.0×10^5 cells/well). Adherent macrophages were isolated by incubating the cells (2 hr at 37°C/5% CO_2), and then, vigorous shaking of plate was done 3 times to wash and remove the non-adherent cells. Cultures were maintained with or without addition of EBP (10, 50, and 100 μg/mL) and LPS (the same concentration as EBP) in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS at 37°C and 5% CO_2 in humidified incubator. Incubation was carried out for 48 hr to determine the production of lysosomal enzyme activity.

Determination of macrophage lysosomal enzyme activity Lysosomal enzyme activity was assayed using 96-well flat-bottomed tissue culture plates (17). Macrophage monolayers in microplate (2.0×10^5 cells/well) were solubilized by the addition of 25 μL of 0.1% Triton X-100. One-hundred-fifty μL of *p*-nitrophenyl phosphate solution (10 mM) was added as a substrate for acid phosphatase. Later, 50 mL of citrate buffer was added to the well, and after incubation (37°C for 1 hr), 25 μL of 0.2 M borate buffer (pH 9.8) was added to the reaction mixture, and the optical density (405 nm) was measured.

Analysis of composition of sugar and amino acid The total sugar content of EBP was measured by the phenol-sulfuric acid method (18), using a mixture of glucose and mannose (5:1) as a standard. The sugar composition was analyzed by a GC 3600 gas chromatograph (Varian Co., Palo Alto, CA, USA), based on hydrolysis and acetylation method described by Jones and Albersheim (19). The total protein content of EBP was measured by the method of Lowry *et al.* (20), with bovine serum albumin as a standard. The amino acid composition in the protein hydrolysate was analyzed by Biochrom 20 amino acid autoanalyzer (Pharmacia Biotech., Stockholm, Sweden).

Statistical analysis Each data value was expressed as the mean±standard error (SE). The group mean value was compared using a one-way analysis of variance (ANOVA) and Duncan's (21) multiple range test. The statistical difference was considered significant at $p<0.05$.

Results and Discussion

Effect on organ weight and tumor inhibition The dose-dependent effect of EBP produced by *P. eryngii* on the immune-related organs and tumor inhibition rate of Sarcoma-180 cell treated BALB/c mice are shown in Table 1. The spleen and liver weights were higher in EBP treated groups than control group. This may be due to an increase in the number of splenic macrophages treated with EBP. Zheng *et al.* (23) reported that the relative spleen weight was important index for nonspecific immunity. It is generally known that splenic macrophage has an immune response to foreign substances in human body and Kupffer cells in liver, by way of production of variety of cytokines including interleukin-1, tumor necrosis factor-α, and superoxides, which are normally stimulated by foreign antigens (24).

The tumor growth was significantly decreased in all the EBP treated groups when compared to control group (Table 1). The maximum inhibition (53.1%) of tumor growth was achieved when the EBP was administered at the level of 40 mg/kg BW. However, the over dose (over 40 mg/kg) of EBP found to be ineffective to the inhibition of tumor growth. Miura *et al.* (22) reported that overdose of β-glucan in Schizophyllan was significantly failed to display the antitumor activity suggesting that the modulation of the cytokine synthesis and the leukocyte traffic may cause the failure of antitumor activity. It has also been reported that the biopolymer including β-glucan isolated from the mycelia and fruiting body of *Pleurotus* sp. is known to have host-mediated antitumor activities (7, 9-11).

Immunomodulating activity The dose-dependent effects

Table 1. Antitumor activities of endo-biopolymer (EBP) produced by *P. eryngii* in Sarcoma-180 cell treated BALB/c mice after 4 weeks

Concentration (EBP, mg/kg BW)	Body weight (g)	Spleen/body weight (%)	Liver/body weight (%)	Tumor weight (g)	Inhibition rate (%)[1)]
Control[2)]	29.7±0.4[c3)]	2.43±0.35[NS]	6.81±0.18[c]	6.43±0.29[c]	-
10	26.9±0.5[a]	2.91±0.07	7.15±0.29[ab]	3.56±0.70[ab]	44.7[ab]
20	26.8±0.3[a]	2.93±0.16	7.39±0.19[a]	3.17±0.15[a]	50.7[ab]
40	27.3±0.6[ab]	2.72±0.16	7.41±0.10[a]	3.01±0.42[a]	53.1[c]
60	27.8±0.6[ab]	2.92±0.15	7.84±0.26[ab]	4.01±0.56[ab]	37.7[ab]
80	28.8±0.6[bc]	2.84±0.14	7.84±0.24[b]	4.78±0.62[b]	25.7[a]

[1)]Inhibition rate=[(Control tumor weight−Treated tumor weight)/Control tumor weight]×100
[2)]Phosphate buffered saline administration
[3)]Each value is mean±SE for 9 mice; [a,b,c]Means in the same column with different superscripts are significantly different ($p<0.05$); [NS]Not significant

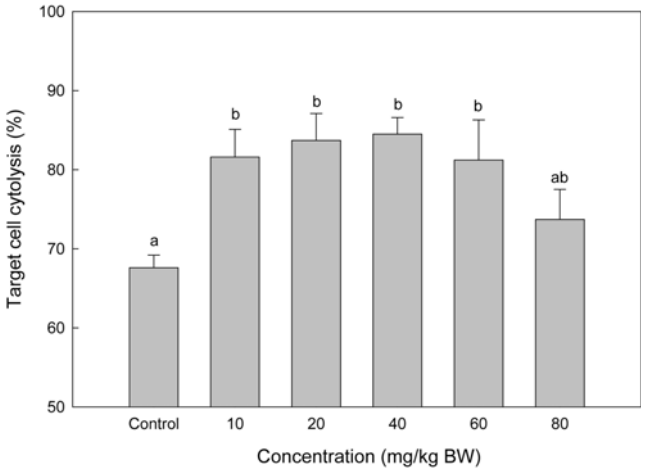

Fig. 1. Effect of endo-biopolymer produced by *P. eryngii* on the natural killer (NK) cell activity of mice splenic lymphocytes in Sarcoma-180 cell treated BALB/c mice after 4 weeks. PBS administration. Each value is mean±SE for 9 mice. [a,b]Means in the same column with different superscripts are significantly different ($p<0.05$).

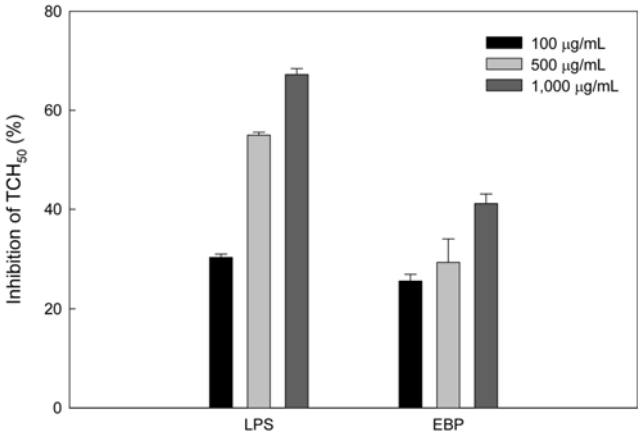

Fig. 2. Anti-complementary activities of the endo-biopolymer (EBP) produced by *P. eryngii*. Lipopolysaccharide (LPS) was used for the positive control. Each value is the mean±SD for triplicates.

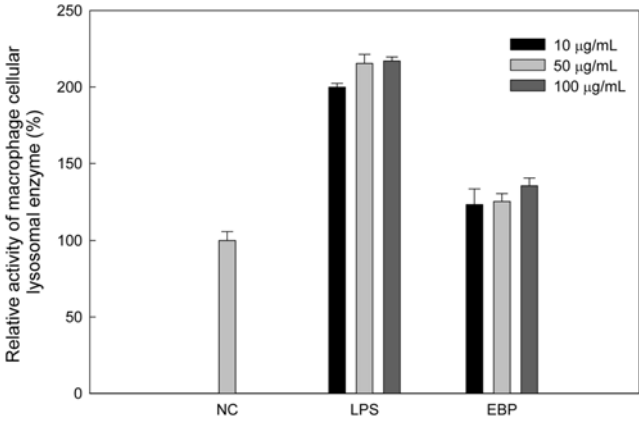

Fig. 3. Macrophage cellular lysosomal enzyme activities of the endo-biopolymer (EBP) produced by *P. eryngii*. Normal saline (NC) was used for the negative control. Lipopolysaccharide (LPS) was used for the positive control. Concentration of macrophage was 2.5×10^6 cells/mL. Each value is the mean±SD for triplicates.

of EBP, on the natural killer (NK) cell activity of mice splenic lymphocytes in sarcoma-180 cell treated BALB/c mice are shown in Fig. 1. The NK cell activity was increased 25.5% when the EBP (40 mg/kg BW) compared with control group. Several investigators have reported that administration of the antitumor polysaccharides or antitumor protein-polysaccharide, isolated from mushrooms, can restore the suppressed NK cell activity in tumor-bearing mice (25,26), thus indicating its important role in the control of tumor growth. Action of NK cells are a subset of lymphocytes which has cytotoxic effects to various malignant and normal cells of the infected host, thus playing an important role in the first-line defense against viral disease and cancer (27,28). The results obtained from the present investigation suggest that EBP produced by *P. eryngii* possess the ability to activate NK cells and thereby it may kill tumor or reduced growth directly.

The complement activity of EBP was compared at the concentrations of 100, 500, and 1,000 μg/mL (Fig. 2). It was found that the complement activity of EBP was increased in accordance with the increase of concentration. The complement activity (41.1%) of EBP was recorded at the concentration of 1,000 μg/mL, thus it indicates that a polymer from mushroom is closely related with antitumor activities by way of activating the complementary system (1,31).

The selective release of lysosomal acid phosphatases (lysosomal enzyme) has also been observed by mononuclear phagocytes, which occur in response to numerous exogenous stimuli (29). In the present study, the influence of the EBP from *P. eryngii* on macrophage lysosomal enzyme activity has been studied at various concentrations (Fig. 3). Treatment with the concentration of 10, 50, and 100 μg/mL of EBP stimulated the enzyme activity upto 23, 25, and 35%, respectively, against negative control (physiological saline). It can be suggested that EBP of *P. eryngii* has macrophage activating capacity to lyse foreign substances engulfed through phagocytosis. Such effect of EBP was found to be similar to that of the biopolymer produced by submerged mycelial culture of *Phellinus pini* (2). It is also recorded that the polysaccharides from the fruiting bodies of *Armillariella tabescens* have antitumor activity on Sarcoma-180, and which was related to lysosomal enzyme activation in macrophages (30,31).

Chemical composition of sugar and protein The total sugar and protein contents of EBP produced during submerged mycelial culture of *P. eryngii* were found to be

Table 2. Sugar and amino acid compositions of endo-biopolymer obtained from *P. eryngii*

Neutral sugar	Composition (M ratio)[1)]	Amino acid	Composition (%)[2)]
Fucose	0.07	Asp	10.2
Ribose	0.33	Thr	6.2
Arabinose	0.09	Ser	8.8
Xylose	0.17	Glu	11.9
Mannose	1.00	Gly	12.9
Galactose	0.26	Ala	9.6
Glucose	5.70	Val	5.8
		Ile	3.2
		Leu	5.2
		Phe	3.9
		His	Trace
		Lys	8.9
		Arg	8.3
Total sugar (%)	77.5	Total protein (%)	21.6

[1)]Calculated on the basis of total sugar and molar mass
[2)]Calculated on on the basis of total amino acid

77.5 and 21.6%, respectively (Table 2). The EBP contained glucose (74.8%) and mannose (13.1%) as the major sugar moiety, while glycine (12.9%), glutamic acid (11.9%), and aspartic acid (10.2%) were the major amino acids. Similar results showing antitumor properties by the polymer isolated from *Elfvingia applanata* contained glucose (89.1%) and mannose (14%), where the sugar and protein contents were 65.3 and 6.5%, respectively (33). Most of the complement enhancer polysaccharide isolated from *Pleurotus ostreatus* contained large amount of glucose (34).

However, a further detailed study is required on the mechanism, by which this complex induces the effect and its additional clinical usefulness in cancer therapies.

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