Activation of Macrophages by Exopolysaccharide Produced by MK1 Bacterial Strain Isolated from Neungee Mushroom, *Sarcodon aspratus*

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**Background:** The MK1 strain, a novel bacterial isolate from soft-rotten tissue of the Neunquee mushroom, produces copious amounts of exopolysaccharide (EPS) in a dextrose minimal medium. This study examined the molecular characteristics and immunomodulatory activity of MK1 EPS. **Methods:** The EPS in the culture supernatant was purified by cold ethanol precipitation, and characterized by SDS-PAGE/silver staining and Bio-HPLC. The immunomodulatory activities of the EPS were examined using the mouse monocytic cell line, RAW 264.7 cells. **Results:** The molecular weights of the purified EPS were rather heterogeneous, ranging from 10.6 to 55 kDa. The EPS was composed of glucose, rhamnose, mannos, galactose, and glucosamine at an approximate molar ratio of 1.00 : 0.8 : 0.71 : 0.29 : 0.21. EPS activated the RAW cells to produce cytokines, such as TNF-α and IL-1β, and nitric oxide (NO). EPS also induced the expression of co-stimulatory molecules, such as B7-1, B7-2 and ICAM-1, and increased the phagocytic activity. The macrophage-activating activity of EPS was not due to endotoxin contamination because the treatment of EPS with polymyxin B did not reduce the macrophage-activating activity. **Conclusion:** The EPS produced from the MK1 strain exerts macrophage-activating activity.

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**INTRODUCTION**

Microbial exopolysaccharides (EPS) are a polycarbohydrate produced and excreted by microorganisms. There are a variety of types of EPS, either homopolysaccharides (e.g. cellulose, dextran, mutan, alternant, pullulan, levan and curdlan) and heteropolysaccharides (e.g. gellan and xanthan) depending on the types of repeating subunit (1). Microbial EPSs are either capsular polysaccharide bound covalently to the cell surface or slime polysaccharides bound loosely to the cell surface (2). Microbial EPSs play a number of indispensable roles in self-protection against harsh environments, such as osmotic stress, desiccation, UV radiation, antimicrobial substances, toxic compounds, and bacteriophage attack (3). In addition, microbial EPSs allow the cells to adhere other bacteria, animal and plant tissues or inert surfaces, thereby forming biofilms (4). Owing to the properties of EPSs, such as water retention, film-forming, texture-enhancing, thickening, water binding, gelling, and suspending, various microbial EPSs are expected to have immense value in detergents, adhesives, heavy metal removal, microbial enhanced oil recovery, wastewater treatment, cosmetics, pharmaceuticals, brewing, food etc. (5). For example, *Enterobacter cloacae* EPS showing good viscosity, even at high temperatures, makes it a good candidate for microbial enhanced oil recovery (6). Gelrite obtained from *Pseudomonas* spp. is a new gelling polysaccharide with good thermal stability and clarity (7). Xanthan gum produced by *Xanthomonas campestris* has a wide range of applications as a thickening and stabilizing agent, particularly in the food industry, cosmetics, paper milling, textiles, and pharmaceutical...
sector as well as in enhanced oil recovery (8). In addition, cell-bound EPS produced by marine bacterium, Zoogloea sp., was reported to adsorb metal ions, such as chromium, lead, and iron in solution (9). In addition, the great value of microbial EPSs would be associated with their various physiological activities, such as anti-tumor, anti-viral, anti-inflammatory, inducer of interferon production, platelet aggregation inhibition, and colony stimulating factor. For example, the EPSs produced by either marine Vibrio or Pseudomonas sp. have anti-tumor, antiviral, and immunostimulating activities (10,11). EPS produced by Bäkteria licheniformis has immunostimulatory activity (15). EPS produced by Lactococcus lactis sp. cremoris KVS20 exhibits bioactivity, such as lymphocyte mitogenecity (16-19), macrophage cytostaticity (20), and cytokine (IFN-κ) production in macrophages (21). The MK1 strain, a bacterial isolate originated from the soft-rotten tissue of the Seung-gill, appears to be a novel bacterium (22,23). The most peculiar feature of the heterotrophic MK1 strain produces copious amounts of EPS in a dextrose medium (24).

In this study, EPS was purified and characterized by SDS-PAGE/silver staining and Bio-HPLC. The purified EPS exhibited immunomodulatory activity on the mouse monocytic cell line.

MATERIALS AND METHODS

Reagents
Carbon sources consumed in minimal salt medium and chemicals, such as acetic acid, formaldehyde, sodium thiosulfate, silver nitrate, and sodium carbonate, used in the silver staining of EPSs were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The size marker kit used to determine the total EPS molecular weight was obtained from Fermentas Inc. (Burlington, Canada). Ethanol for EPS purification and sulfu-ric acid needed to determine the glucose equivalent EPS were supplied by Merck Ltd. (Bangkok, Thailand). Alcian blue (a cationic copper phthalocyanine dye) binds to the anionic carboxyl and half-ester sulfate groups of acidic EPS to form an insoluble precipitate. The intensity of the blue color of the supernatant is inversely proportional to the amount of acidic EPS in the reaction mixture, Xanthan gum, the reference material needed to assay the acidic sugar quantitation in EPS, was dissolved in D-H2O to 0.5 mg/ml by stirring with mild heating for 30 min and then stored at 4°C. Using a standard curve con-

Purification of MK1 exopolysaccharides (EPS)
The supernatant containing EPS was obtained from the MK1 cell culture by centrifugation (SUPRA 22K, Hanil, Korea) at 6,520×g for 20 min at 4°C. The EPS in the supernatant was precipitated with three volumes of absolute cold ethanol followed by incubation at 4°C for 24 hr. The ethanol precipitated EPS was collected by centrifugation for 15 min at 4,520×g, at 4°C, and the EPS precipitate was suspended in 2 ml of D-H2O. The EPS was washed three times with cold ethanol and freeze-dried (Ilsin® Lab., Korea) at −50°C for 2 days (25). This freeze-dried EPS was used for the morphological observations by optical microscopy and scanning electron microscopy (SEM, S-2500C, Hitachi, Japan). The dried EPS was adjusted with D-H2O to a final volume of 5 ml and dialyzed (M.W. cut off 1,000 Da) against 36 L of D-H2O for 3 days at 4°C with four changes per day. The EPS in the dialyzed preparation was recovered by cold ethanol precipitation and freeze-drying, as described above. The dried EPS was used for the EPS quantitation, sugar composition analysis, etc.

EPS quantitation
To express the quantity of EPS, both amounts of glucose (glucose-equivalent EPS) and acidic sugar (acidic sugar equivalent EPS) were assayed using the phenol-sulfuric acid method (26,27) and alcian blue binding (25,28), respectively. The glucose-equivalent EPS was determined using the standard curve constructed with different glucose concentrations (100, 200, 300, 400, 500 μg/ml). Alcian blue (a cationic copper phthalocyanine dye) binds to the anionic carboxyl and half-ester sulfate groups of acidic EPS to form an insoluble precipitate. The intensity of the blue color of the supernatant is inversely proportional to the amount of acidic EPS in the reaction mixture, Xanthan gum, the reference material needed to assay the acidic sugar quantitation in EPS, was dissolved in D-H2O to 0.5 mg/ml by stirring with mild heating for 30 min and then stored at 4°C. Using a standard curve con-
structed with different xanthan gum concentrations (50, 100, 150, 200 μg/ml), the Y-axis on the standard curve was calculated by subtracting the OD600 for each xanthan concentration from a blank. The quantity of acidic sugar in EPS was estimated from the standard curve. All analyses were performed in triplicate.

Resolution of EPS by SDS-PAGE
The freeze-dried EPS was resolved by SDS-polyacrylamide gel electrophoresis (29). EPS was dissolved in a buffer containing 2% SDS, 25% glycerol, 60 mM Tris-HCl (pH 6.8), 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue to a concentration of 1% (w/v), and heated to 100°C for 15 min. SDS-PAGE were carried out on 0.1% SDS-15% polyacrylamide gel (100×80×0.15 mm) in the electrophoresis buffer (pH 8.8) containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The details to visualize EPS on the gel by silver staining can be found in reference (30). The molecular weights were estimated using the Rf values of the EPS bands from a standard curve constructed with the Rf values (the relative mobility) of the protein size markers, which were plotted on a semi-logarithmic graph as a function of the protein molecular weights (31).

Analysis of MK1 EPS composition
The sugar composition was analyzed by Bio-liquid chromatography at the Carbohydrate Bioproduct Research Center of Sejong University (Seoul, Korea). Twenty μg of the freeze-dried EPS was hydrolyzed at 100°C for 4 hr with 2 M trifluoroacetic acid in sealed test tubes. The EPS-acid hydrolysates were analyzed on a CarboPacTM PA1 column with an HPAEC-PAD system (Dionex, Sunnyvale, CA, USA). The column was eluted at a flow rate of 1 ml/min with 18 mM NaOH (for neutral and amino sugar detection) and 100 mM NaOH/150 mM NaOAc (for acidic sugar detection), respectively.

Cell culture
The RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Hyclone Laboratories Inc., Logan, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) 100 U/ml penicillin and 100 μg/ml streptomycin (Hyclone), and 50 μM 2-mercaptoethanol (SIGMA, St. Louis, MO, USA) at 37°C in an atmosphere containing 5% CO₂.

Proliferation assay
The RAW cells were cultured in the presence of different EPS concentrations (48.32 μg glucose-equivalent EPS/mg EPS, 9.0 μg acidic sugar equivalent EPS/mg EPS) in a 96-well microtiter plate (2×10⁴ cells/well). DNA synthesis was measured by the level of [³H]-thymidine (2 Ci/mmol, PerkinElmer, Shelton, CT, USA) incorporation at a concentration of 0.5 μCi/well for the final 6 hr of the 2 day culture period. The cells were harvested onto glass fiber filter paper using an automated cell harvester (Inotech, Dottikon, Switzerland). The filters were washed, dried and then counted in a microbeta liquid scintillation counter (Wallac, Turku, Finland).

Cytokine production
The RAW cells were cultured in the presence of different EPS concentrations in a 24-well microtiter plate (5×10⁵ cells/well) in a total volume of 1 ml. After 48 hr incubation, the culture supernatants were collected, and the quantities of IL-1β and TNF-α were measured using commercial immunoassay kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instruction.

Nitric oxide production
The RAW cells were cultured in the presence of different concentrations of EPS in a 24-well microtiter plate (5×10⁵ cells/well) in a total volume of 1 ml. After 48 hr stimulation, 50 μl of the cell-free supernatants were collected, incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, 5% H₃PO₄) at room temperature for 5 min, and the absorbance at 550 nm was determined using a VERSAmax (Molecular Devices Inc., CA, USA) microplate reader. The NO₂⁻ concentration was determined from the least squares linear regression analysis of a sodium nitrite standard curve.

Flow cytometry
The cells were stained with the monoclonal antibodies recognizing murine cell surface markers, as previously described (32). Phenotypic analysis of the macrophages was performed on a FACS™ Canto II (Becton-Dickinson, San Jose, CA, USA), and the data was analyzed using FlowJo software. The monoclonal antibodies, anti-CD40 (clone 3/23), anti-ICAM-1 (clone 3E2), anti-I-Ab (clone AF6-120.1), anti-B7-1 (clone 16-10A1), anti-B7-2 (clone GL1), and isotype-matched control antibodies were purchased from Pharmingen (San Diego, CA, USA). The dead cells were gated out by their low forward angle light
scatter intensity. In most analyses, 10,000 cells were scored.

**Phagocytic activity**
The RAW cells were cultured in 6-well plates (2×10^6 cells/well) in the presence or absence of EPS for 2 days, and then added with biodegradable microspheres (average diameter, 300 nm) containing both ovalbumin (OVA) and fluorescein isothiocyanate (FITC). The cells were incubated for 2 hr and washed with pre-warmed PBS to remove the unphagocytozed microspheres. The cells were then harvested by pipetting after cooling on ice for 20 min, fixed in 1% paraformaldehyde in PBS, and analyzed by flow cytometry on a FACSCanto II. Microspheres containing both OVA and FITC were prepared using a solvent-evaporation method (33). Briefly, OVA was dissolved in 3% polyvinyl alcohol (final, 4 mg/ml), and poly (l-lactic acid) (PLG, final, 5%), and FITC (final, 5 mg/ml) were dissolved in a mixture of acetone and ethanol (9:1). These two solutions, 150 ml of OVA solution and 30 ml of the PLG solution, were mixed slowly and emulsified by continuous stirring overnight at room temperature. The hardened microspheres were collected by centrifugation at 300×g, and washed twice with PBS.

**Removal of endotoxin**
The possible contaminants of endotoxin contained in the EPS were removed using Affi-Prep Polymyxin Matrix (BIO-RAD, Hercules, CA, USA). Briefly, 1 ml of Affi-Prep Polymyxin Matrix was packed in a Bio-spin column (BIO-RAD), centrifuged for 2 min at 200×g, and then 0.5 ml of the EPS (200 μg/ml) was added. After incubating overnight at 4°C, the effluent was recovered from the column by centrifugation under the same conditions.

**RESULTS**

**Composition and characterization of MK1 EPS**
The EPS obtained from the MK1 strain grown in a minimal glucose medium was observed by optical microscopy and SEM (Fig. 1). The freeze-dried EPS was a white powder and was readily soluble in water. The molecular weights of the purified EPS were rather heterogeneous, ranging from 10.6 to 55 kDa, when it was resolved by SDS-PAGE/silver staining (Fig. 2). Complete hydrolysis of the EPS with 2 M trifluoroacetic acid followed by monosaccharide composition analysis showed that this EPS was a heteropolysaccharide composed of glucose, rhamnose, mannose, galactose, glucosamine in an approximate molar ratio of 1.00 : 0.8 : 0.71 : 0.29 : 0.21 with a trace amount of fucose, fructose, and galacturonic acid, as shown in Fig. 3.

**Effects of MK1 EPS on the growth of RAW cells**
To examine the immunomodulatory activity of EPS, EPS was added to the cultures of RAW cells, and the growth inhibitory activity was observed 2 days later. The growth inhibitory ac-
tivity of the EPS on RAW cells was documented further by the \[^{3}H\]-thymidine uptake for the final 6 hr of the 2 day culture period. As shown in Fig. 4, EPS inhibited the growth of RAW cells in a dose dependent manner. The growth inhibitory activity of EPS reached 90% at 100 \(\mu\)g/ml. EPS was treated with polymyxin B, which is a specific inhibitor of polysaccharide, to ensure that the effects of the EPS were not due to endotoxin contamination. Passage of the EPS solution (200 \(\mu\)g/ml) through the polymyxin B-affinity column did not reduce the growth inhibitory activity. The growth inhibitory activity of EPS was not due to direct cytotoxicity.

**Effects of MK1 EPS on the expression of surface molecules**

The increased expression of co-stimulatory molecules can be a marker of macrophage activation. Therefore, the effects of EPS on the expression of co-stimulatory molecules on RAW cells, which were involved in T cell activation, were also examined. As shown in Fig. 5, 100 \(\mu\)g/ml EPS increased slightly the expression of B7-1, B7-2 and ICAM-1. This demonstrates that EPS induces the further differentiation of macrophages.

**Effects of MK1 EPS on the production of cytokines and nitric oxide**

Cytokine and nitric oxide production is a parameter of the functional activation of macrophages. To determine if the EPS-activated RAW cells produced cytokines and nitric oxide, the culture supernatants were collected at 48 hr and the quantity of TNF-\(\alpha\), IL-1\(\beta\) and nitric oxide were measured. The stimulatory activity of EPS on macrophages was demonstrated further by the dose-dependent increase in TNF-\(\alpha\) production (Fig. 6A), IL-1\(\beta\) production (Fig. 6B) and nitric oxide release (Fig. 6C) by RAW cells stimulated with EPS. As shown in a representative result in Fig. 6, the incubation of EPS with a polymyxin B-affinity column did not reduce the macrophage-activating activity.

**Effects of MK1 EPS on phagocytic activity**

The phagocytic activity of the EPS-activated RAW cells was examined using the microspheres containing fluorescein isothiocyanate (FITC). One of the most distinguishing features of macrophage activation would be the increase in phagocytic activity. RAW cells were cultured in the presence of EPS (100 \(\mu\)g/ml) for 2 days, and added with the microspheres contain-
Figure 5. Phenotypic analysis of RAW cells stimulated with MK1 EPS. EPS was treated with a polymyxin B-affinity column to remove the possible contaminants of endotoxin. The RAW cells were cultured in the presence of EPS (100 μg/ml) for 2 days. The cells were collected, washed and used for immunophenotypic analysis. The levels of expression (thin line) are shown along with the isotype control (shaded line).

Figure 6. Cytokine and nitric oxide production of RAW cells stimulated with MK1 EPS. EPS was treated with a polymyxin B-affinity column to remove any possible endotoxin contamination. The RAW cells were cultured in the presence of different MK1 EPS concentrations (0.8, 4, 20 and 100 μg/ml) for 2 days, and the culture supernatants were assayed for TNF-α (A), IL-1β (B). The amounts of nitric oxide were measured using a Griess reagent (C).

The cells were incubated for 2 hr and washed with pre-warmed PBS to remove the unphagocytozed microspheres. The cells were harvested, fixed and analyzed by flow cytometry. As shown in Fig. 7, the thin line histograms represent the phagocytic activity of RAW cells stimulated with EPS, and the shaded histograms represent the phagocytic activity of the untreated RAW cells. EPS activated the phagocytic activity. This demonstrates that EPS is an activator of...
Figure 7. Phagocytic activity of RAW cells stimulated with MK1 EPS. EPS was treated with a polymyxin B-affinity column to remove any possible endotoxin contamination. The RAW cells were cultured in the presence of EPS (100 μg/ml) for 2 days, followed by the addition of microspheres containing fluorescein isothiocyanate (FITC). After 2 hr, unphagocytozed microspheres were removed by washing. The cells were harvested, fixed, and analyzed by flow cytometry. The thin line histograms represent the phagocytic activity of the RAW cells stimulated with MK1 EPS and the shaded histograms represent the phagocytic activity of the untreated RAW cells.

Macrophages. Passage of the EPS solution (200 μg/ml) with the polymyxin B-affinity column did not reduce the phagocytic activity of RAW cells.

DISCUSSION

These results show that the EPS produced by the MK1 strain isolated from Neungee mushroom is an immunomodulatory polysaccharide with no cytotoxic effects. EPS was shown to inhibit the proliferation of RAW cells, inducing morphological changes from slightly adherent monocytic cells to strongly adherent macrophages. Because macrophages are end stage cells that do not proliferate further, the growth inhibitory activity of EPS may be due to the induction of further differentiation of monocytic RAW cells. EPS was also shown to stimulate RAW cells to produce nitric oxide, which is the principal effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation (34). EPS stimulated the RAW cells to produce TNF-α and IL-1β, which have often been implicated as key mediators produced from macrophages in response to bacterial LPS, infection and inflammatory stimuli (35,36).

Immature macrophages do not induce primary immune responses because they do not express the requisite class II MHC molecules and co-stimulatory molecules, nor do they express antigenic peptides as stable complexes with MHC molecules. EPS was shown to enhance the expression of co-stimulatory molecules, such as B7-1 and B7-2, and class II MHC molecules. One of the most important parameters of macrophage activation is the increased phagocytic activity. Phagocytosis is an innate immune response mechanism for the removal of foreign pathogens, EPS was shown to activate the phagocytic activity on macrophages. These results show that EPS is a good activator of macrophages.

Indeed, plant-derived polysaccharides have been implicated for immunostimulatory polysaccharides. For example, immunostimulatory polysaccharides have been isolated from Aloe vera (32,37), Angelica acutiloba (38), Coriolus versicolor (39), Schizophyllum commune (40), and Lentinus edodes (41). The immunostimulatory polysaccharides isolated from these plants or mushrooms appear to trigger the immune responses primarily by activating macrophages, even though the direct activation of B cells and other immune cells has been implicated. Composition analysis of the polysaccharide suggests that it is quite different from well known immunomodulatory polysaccharides, such as acemannan, lentinon and PSK (37,39,41). Possible contamination of endotoxin is always a concern for high molecular weight components because endotoxin is a strong activator of macrophages and is contaminated in many plant materials. In this study, the macrophage-activating activity of EPS was not due to endotoxin contamination, as shown by polymyxin B-treatment experiments. A particular component of microbial EPS seems to be engaged in specific bioactivity. For example, phosphate-containing EPS produced by Lactobacillus delbrueckii sp. bulgaricus induced macrophage activation (42). Mannose-rich EPS from Tremella mesenterica (43) or rhamnose-rich (6-deoxy-L-mannose) EPS from Lactobacillus rhamnosus (44)
stimulated the immune system through the mannose receptors situated on macrophage. Fucosamine-containing EPS produced by a marine *Pseudomonas* exhibited strong antiviral activity (11). L-fucose-enriched EPS would be used as a skin moisturizing agent in the cosmetic industry because L-fucose exhibited anticancer and anti-inflammatory activity (45). Sulfate-containing EPS showed a broad range of important biocactivities, such as antioxidant, anticoagulant, and anti-thrombotic activities (46). Therefore, it is possible that macrophage activation by EPS would be associated with the rhamnose or mannose moieties in its structure.

Macrophages are the most important defense cells in the immune system in that they not only initiate immune responses, but can also serve as effector cells. Furthermore, macrophages are unique components of the innate immunity and have a range of functions related to the activation process. Activated macrophages become more efficient antigen-presenting cells because they express increased levels of class II MHC molecules and co-stimulatory molecules (47), altered phagocytic activity as well as increased cytokine and nitric oxide production (48). Because EPS exerts strong macrophage-activating activity and the MK1 strain produces copious amounts of EPS, these results may lead to the discovery of a new source of immunomodulatory polysaccharides that can be obtained in large quantities.

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CONFLICTS OF INTEREST

The author have no financial conflict of interest.

REFERENCES

1. Welman AD, Maddox IS: Exopolysaccharides from lactic acid bacteria: perspectives and challenges, Trends Biotechnol 21:269-274, 2003
2. Sutherland IW: Bacterial exopolysaccharides, Adv Microb Physiol 8:145-213, 1972
3. Wang H, Jiang X, Mu H, Liang X, Guan H: Structure and protective effect of exopolysaccharide from P. Agglomeras strain KPS-9 against UV radiation, Microbiol Res 162:124-129, 2007
4. Sutherland I: Biofilm exopolysaccharides: a strong and sticky framework, Microbiology 147:3-9, 2001
5. Kumar GG, Joo HS, Choi JW, Koo YM, Chang KS: Purification and characterization of an extracellular polysaccharide from halophilic *Bacillus* sp. 1-150, Enzyme Microb Technol 34:673-680, 2004
6. Iyer A, Mody K, Jha B: Rheological properties of an exopolysaccharide produced by a marine *Enterobacter cloacae*, Natl Acad Sci Lett 28:119-123, 2005
7. Lin CC, Gasida LE: GELRITE as a gelling agent in media for the growth of thermophilic microorganisms, Appl Environ Microbiol 47:427-429, 1984
8. Kalogiannis S, Iakovidou G, Liakopoulou-Kyriakides M, Kyriakidis DA, Skaracis GN: Optimization of xanthan gum production by *Xanthomonas campestris* grown in molasses, Process Biochem 39:249-256, 2003
9. Kong J, Lee H, Hong J, Kang Y, Kim J, Chang M, Bae S: Utilization of a cell-bound polysaccharide produced by the marine bacterium *Zooglea* sp: new biomass for metal adsorption and enzyme immobilization, J Mar Biotechnol 6:99-103, 1998
10. Okutani K: Antitumor and immunostimulant activities of polysaccharides produced by a marine bacterium of the genus Vibrio, Bull Jap Soc Fish 50:1035-1037, 1984
11. Okutani K: Antiviral activities of sulfated derivatives of a fucosamine-containing polysaccharide of marine bacterial origin, Nippon Suisan Gakkai 58:927-930, 1992
12. Abbad Andaloussi S, Talbaoui H, Marczak R, Bonaly R: Isolation and characterization of extracellular polysaccharides produced by *Bifidobacterium* longum, Appl Microbiol Biotechnol 43:995-1000, 1995
13. Roberts CM, Felt WF, Osman SF, Wijey C, O'Connor JV, Hoover DG: Exopolysaccharide production by *Bifidobacterium* longum BB-79, J Appl Bacteriol 78:643-648, 1995
14. Sreekumar O, Hosono A: The antimutagenic properties of polysaccharides produced by *Bifidobacterium* longum and its cultured milk against some heterocyclic amines, Can J Microbiol 44:1029-1036, 1998
15. Arena A, Maugeri TL, Pavone B, Innello D, Gugliandolo C, Bisignano G: Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis*, Int Immunopharmacol 6:2003-2006
16. Kitaizawa H, Yamaguchi T, Itoh T: B-cell mitogenic activity of a novel exopolysaccharide from a marine bacterium *Bacillus licheniformis*, FEBS Lett 295:1, 1992
17. Kitaizawa H, Yamaguchi T, Miura M, Saito T, Itoh T: B-cell mitogenic activity of slime products produced from slime-forming, encapsulated *Lactobacillus lactis* spsp., Cremonis, J Dairy Sci 75:2946-2951, 1992
18. Kitaizawa H, Yamaguchi T, Miura M, Saito T, Itoh T: B-cell mitogenic activity of slime products produced from slime-forming, encapsulated *Lactobacillus lactis* spsp., Cremonis, J Dairy Sci 76:1514-1519, 1993
19. Kitaizawa H, Yamaguchi T, Fujimoto Y, Itoh T: Comparative activity of B-cell mitogen, a phosphopolysaccharide, produced by *L. lactis* spsp., Cremonis on various lymphocytes, Anim Sci Technol 64:605-607, 1993
20. Kitaizawa H, Yamaguchi T, Fujimoto Y, Itoh T: Analysis of mitogenic response of phosphopolysaccharide, a B-cell mitogen produced by *Lactobacillus lactis* spsp., Cremonis to...
spleen cells, Anim Sci Technol 64:807-812, 1993
20. Kitazawa H, Itoh T, Yamaguchi T: Induction of macrophage cytotoxicity by slime products produced by encapsulated Lactococcus lactis ssp. cremoris, Anim Sci Technol 62:861-866, 1991
21. Kitazawa H, Itoh T, Tomioka Y, Mizugaki M, Yamaguchi T: Induction of IFN-γ and IL-1α production in macrophages stimulated with phosphopolysaccharide produced by Lactococcus lactis ssp. cremoris, Int J Food Microbiol 31:99-106, 1996
22. Lee YN, Koo CD: Identification of bacteria isolated from diseased Neungge mushroom, Sarcodon aspratus, J Basic Microbiol 47:31-39, 2007
23. Lee YN, Kim EJ: Characterization of a novel bacterium isolated from diseased Neungge mushroom, Sarcodon aspratus, 11th Int Sym on the Genetics of Industrial Microorganisms, Melbourne, Australia: Abs, p29, 2010
24. Ryu JE, Lee YN: Optimizing culture condition of MK1 strain isolated from soft-rotten tissue of Neungge mushroom and its exopolysaccharide, Korean J Microbiol 45:324-331, 2009
25. McKellar RC, Geest JV, Cui W: Influence of culture and environmental conditions on the composition of exopolysaccharide produced by Agrobacterium radiobacter, Food Hydrocolloids 17:429-437, 2003
26. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F: A colorimetric method for the determination of sugars, Nature 166:268-269, 1951
27. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura SI, Lee YC: Carbohydrate analysis by a phenol-sulfuric and colormetric method for the determination of sugars, Nature 168:167, 1951
28. Ramus J: Alcian blue: A quantitative aqueous assay for algal and sulphated polysaccharides, J Physiol 13:345-348, 1977
29. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:680-685, 1970
30. Kim JB, Ahn JE: The modification of the silver stain method in sodium dodecyl sulfate-polyamide gels for detecting lipopolysaccharides, J Korean Soc Microbiol 28;193-198, 1993
31. Hedrick JL, Smith AJ: Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis, Arch Biochem Biophys 126:155-164, 1968
32. Lee JK, Lee MK, Yun Y-P, Kim Y, Kim JS, Kim YS, Kim K, Han SS, Lee CK: Acamamin purified from Aloe vera induces phenotypic and functional maturation of immature dendritic cells, Int Immunopharmacol 1:1275-1284, 2001
33. Gerechtshaul T, Lee YH, Lee YR, Im SA, Song S, Park JS, Han K, Kim K, Lee CK: Dendritic cell process antigens encapsulated in a biodegradable polymer, poly (D,L-lactide-co-glycolide), via an alternate class I MHC processing pathway, Arch Pharm Res 30:1440-1466, 2007
34. Lorschub RB, Murphy WJ, Lowenstein CJ, Snyder SH, Russell SW: Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing, Molecular basis for the synergy between interferon-gamma and lipopolysaccharide, J Biol Chem 268:1908-1913, 1993
35. Sherwin C, Fern R: Acute lipopolysaccharide-mediated injury in neonatal white matter glia: role of TNF-alpha, IL-beta, and calcium, J Immunol 175:155-161, 2005
36. Shamsh S, Reichert F, Rothshenker S: The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1α, and interleukin-1β, J Neurosci 22:3052-3060, 2002
37. Im SA, Oh ST, Song S, Kim MR, Kim DS, Woo SS, Jo TH, Park YI, Lee CK: Identification of optimal molecular size of modified Aloe polysaccharides with maximum immunomodulatory activity, Int Immunopharmacol 5:271-279, 2005
38. Yamada H, Kiyohara H, Cyong JC, Kojima Y, Kumazawa Y, Osuka Y: Studies on polysaccharides from Angelica architita, Part I, Fractionation and biological properties of polysaccharides, Planta Med 50:163-167, 1984
39. Pang ZJ, Zhou M, Chen Y, Wan J: A protein-bound poly saccharide synergistic with lipopolysaccharide induces nitric oxide release and antioxidant enzyme activities in mouse peritoneal macrophages, Am J Chin Med 26:133-141, 1998
40. Okamura K, Suzuki M, Yajima A, Chihara T, Fujivara A, Fukuda T, Goto S, Ichinohoe K, Jimi S, Kasamatsu T, Kawai N, Mizuguchi K, Mori S, Nakano H, Noda K, Sekiha K, Suzuki K, Suzuki T, Takakushi K, Takeuchi K, Takeuchi S, Ogawa N: Clinical evaluation of schizophyllan combined with irradiation in patients with cervical cancer, A randomized controlled study, Cancer 58:865-872, 1986
41. Sasuki T, Takasaka N: Further study of the structure of lentinian, an anti-tumor polysaccharide from Lentinus edodes, Carbohydr Res 47:99-104, 1976
42. Kitazawa H, Harata T, Uemura J, Suito T, Kaneko T, Itoh T: Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from Lactobacillus delbrueckii subsp. bulgaricus, Int J Food Microbiol 40:169-175, 1998
43. Chen NY, Hsu TH, Lin FY, Lai HH, Wai JW: Effects on cytokine-stimulating activities of EPS from Tremella mesenterica with various carbon sources, Food Chem 99:392-97, 2006
44. Chabot S, Yu HL, Lesleuc LD, Clotier D, Van Calsteren MR, Lessard M, Van Calsteren MR, Lessard M, Roy D, Larcoix M, Oth D: Exopolysaccharides from Lactobacillus rhamnosus RW-9959M stimulate TNF-α, IL-6 and IL-12 in human and mouse cultured immunocompetent cells and IFN-γ in mouse splenocytes, Lait 81:683-697, 2001
45. Gessutti P, Kallioinen A, Impallomeni G, Toffanin R, Pollesello P, Leisola M, Eerikainen T: Structure of exopolysaccharide produced by Enterobacter amnigenus, Carbohydr Res 340:439-447, 2005
46. Castro R, Piazzon MC, Zarra I, Leiro J, Noya M, Lamas J: Stimulation of turbot phagocytes by Ulva rigida C. Agardh Stimulation of turbot phagocytes by Ulva rigida C. Agardh polysaccharides, Aquaculture 254;9-20, 2006
47. Adams DO, Hamilton TA: The cell biology of macrophage activation, Annu Rev Immunol 2:283-318, 1984
48. Fujivara N, Kobayashi K: Macrophages in inflammation, Curr Drug Targets Inflamm Allergy 4:281-286, 2005