J. Microbiol. Biotechnol. (2009), **19**(6), 566–572
doi: 10.4014/jmb.0809.541
First published online 29 January 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Study on Immunostimulating Activity of Macrophage Treated with Purified Polysaccharides from Liquid Culture and Fruiting Body of *Lentinus edodes*

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Received: September 22, 2008 / Revised: October 30, 2008 / Accepted: November 10, 2008

***Lentinus edodes* is a well-known edible and medicinal mushroom used in Oriental cultures. Recently, *L. edodes* has attracted a lot of attention owing to its antifungal activity, antibacterial activity, antiviral activity, hepatoprotective effect, antitumor activities, and immunomodulatory and cytotoxic effects. In this study, the water-soluble crude polysaccharides, CPF and CPB, which were obtained from the fruiting body and culture cell-free broth of *L. edodes* by hot-water extraction and ethanol precipitation, were fractionated by DEAE cellulose and Sepharose CL-6B column chromatography, resulting in six polysaccharide fractions, CPFN-G-I, CPFN-G-II, CPFN-G-III, CPFA-G, CPBN-G, and CPBA-G. Among these fractions, CPFN-G-I, CPBN-G, and CPBA-G were shown to stimulate the functional activation of macrophages including NO production, cytokine expression, and phagocytosis.**

Keywords: *Lentinus edodes*, immunostimulating polysaccharide, mushroom-derived β-glucan, macrophage activation, phagocytosis

Most, if not all, Basidiomycetes mushrooms have biologically active polysaccharides in the fruiting body, culture cell-free broth, and cultured mycelium. Polysaccharides isolated from mushrooms are the best known and most potent antitumor macromolecules with immunomodulating properties. These polysaccharides have a different chemical composition than most other polysaccharides and primarily belong to the β-glucan group [2, 4, 34, 37, 43]. Calvacin was isolated for the first time from *Calvatia gigantean* in a study that examined the antitumor activities of extracts from Basidiomycetes mushrooms [15, 36]. These findings subsequently led to the development of therapeutic drugs that were derived from medicinal mushrooms. Several of these drugs consisted of the following purified polysaccharides, specifically β-glucans; schizophyllan from the liquid cultured broth products of *Schizophyllum commune*, PS-K from the cultured mycelia of *Coriolus versicolor*, and lentinan from the fruiting body of *Lentinus edodes* [7, 23, 42]. Many studies have demonstrated that the polysaccharides from Basidiomycetes mushroom had highly beneficial therapeutic effects including (1) preventing oncogenesis after administering of peroral medications that were from these mushrooms or their extracts, (2) direct antitumor activity against various tumors, (3) immunosynersim activity against tumors in combination with chemotheraphy, and (4) preventive effects on tumor metastasis [8, 10, 32, 45].

Over the past few decades, extensive studies have been conducted to determine the mechanism by which macrophages kill tumor cells. In a direct manner, the function of activated macrophages is to recognize and kill tumor cells. However, they also play an indirect role in antitumor activity by secreting secondary compounds, such as tumor necrosis factor (TNF) and nitric oxide (NO), that are harmful to cancer cells and by regulating the immune system to process and present antigens [28]. TNF, which is produced by activated macrophages, is able to kill tumor cells by binding to TNF-receptors. Recently, TNF-related apoptosis-inducing ligand (TRAIL) and the Fas-ligand (FasL), another member of the TNF family, were shown to be related to tumor cell death [16, 21, 39]. NO is synthesized enzymatically from L-arginine by NOS in activated macrophages. The toxic effects of NO on tumor cells are due to the inactivation of iron-sulfur cluster-containing enzymes, which inhibits the DNA-binding activity of zinc finger-type transcription factors and destroys the mitochondrial membrane potential [24].

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Several studies are currently being conducted on polysaccharides purified from the fruiting body of *Lentinus edodes*; however, most of these studies have primarily focused on understanding the structure and efficacy of these extracted polysaccharides. Therefore, the aim of this study was to better understand and characterize the immunostimulating activities of polysaccharides that have been isolated and purified from the fruiting body and culture cell-free broth of *Lentinus edodes* by gel filtration chromatography and ion-exchange chromatography. This was achieved by investigating phagocytic uptake, the release of NO, and the production of TNF-α in macrophages, which are in charge of the innate immune response, that were activated by these polysaccharides.

MATERIALS AND METHODS

Materials

Lentinus edodes KCTC 6734 was grown in liquid containing 60 g/l glucose, 10 g/l yeast extract, 1.0 g/l $MgSO_4 \cdot 7H_2O$, and 2.0 g/l KH_2PO_4 in a 5 l bioreactor at 24°C, 300 rpm, and 1.5 vvm. Exopolysaccharide fractions (CPB) derived from the liquid cultures of *L. edodes* were prepared using the ethanol precipitation and lyophilization methods. The polysaccharide fraction (CPF) derived from the fruiting body of *L. edodes* was prepared using ethanol precipitation and lyophilization after hot-water extraction. Polysaccharides, CPB and CPB, were further purified by fractional precipitation using ion-exchange chromatography with DEAE cellulose and gel filtration chromatography with Sepharose CL-6B; namely, CPFN, CPFN-G-I, CPFN-G-II, CPFN-G-III, CPFA, CPFA-G, CPBN, CPBN-G, CPBA, and CPBA-G. These fractions contained an endotoxin level that was below the detection limit (0.0015 EU/ml) as assessed using an E-TOXATE kit (Sigma, St. Louis, MO, U.S.A.). FITC-dextran and lipopolysaccharide (LPS; *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum and RPMI1640 were obtained from Gibco (Grand Island, NY, U.S.A.). RAW264.7 cells were purchased from the American Tissue Culture Center (Rockville, MD, U.S.A.). All other chemicals were of Sigma grade.

Determination of Molecular Mass

The molecular mass of the polysaccharide fractions was determined by gel filtration using the Sepharose CL-6B packed column. A

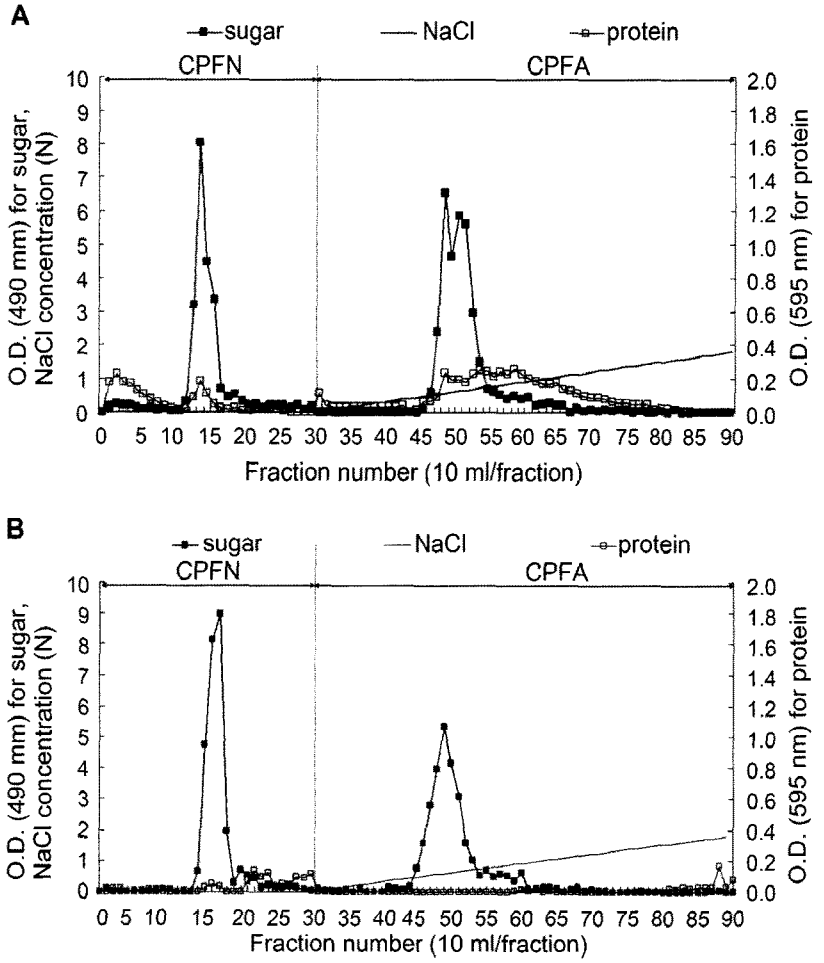

Fig. 1. Ion-exchange chromatograms of crude polysaccharides on a DEAE cellulose column (2.5 × 50 cm).
A. Chromatogram of the crude polysaccharide extracted from the fruiting body (CPF). **B.** Chromatogram of crude polysaccharide extracted from the cultured cell-free broth.

standard curve was prepared based on the elution volume and the molecular mass. Standard dextrans (MW: 670 kDa, 410 kDa, 150 kDa, 25 kDa; Sigma Chemical Co., St. Louis, MO, U.S.A.) were used for calibration.

Chemical Properties Analysis

The total sugar content of each polysaccharide was measured using the phenol-sulfuric acid method [6] and total protein concentration was determined using the Bradford method [3].

Cell Culture

RAW264.7 cells were maintained in RPMI1640 that was supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Cell Viability

The effect of polysaccharides on the viability of the RAW264.7 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells [5]. After pre-incubating RAW264.7 cells (1×10^6 cells/ml) for 18 h, CPFN-G-I, CPBN-G, CPBA-G (100 μg/ml), or LPS (2.5 μg/ml) was added and incubated with the cells for an additional 24 h, as reported previously [22]. Fifty μl of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 μl. After incubation for 4 h, the plate was centrifuged at 800 ×*g* for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide and the A_{540} was read on a scanning multiwell spectrophotometer.

Determination of Phagocytotic Uptake

A previously described method, with slight modifications, was used to measure the phagocytic activity of RAW264.7 cells [12]. RAW264.7 (2×10^6) cells were resuspended in 1 ml of PBS containing 1% human AB serum and incubated with FITC-dextran (1 mg/ml) in the presence or absence of LPS (2.5 μg/ml) or each polysaccharide (100 μg/ml) at 37°C for 30 min. Phagocytosis was then stopped by adding 2 ml of ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

Determination of NO Production

After pre-incubating the RAW264.7 cells (1×10^6 cells/ml) for 18 h, CPFN-G-I, CPBN-G, CPBA-G (100 μg/ml), or LPS (2.5 μg/ml) was added and incubated with the cells for an additional 24 h, as

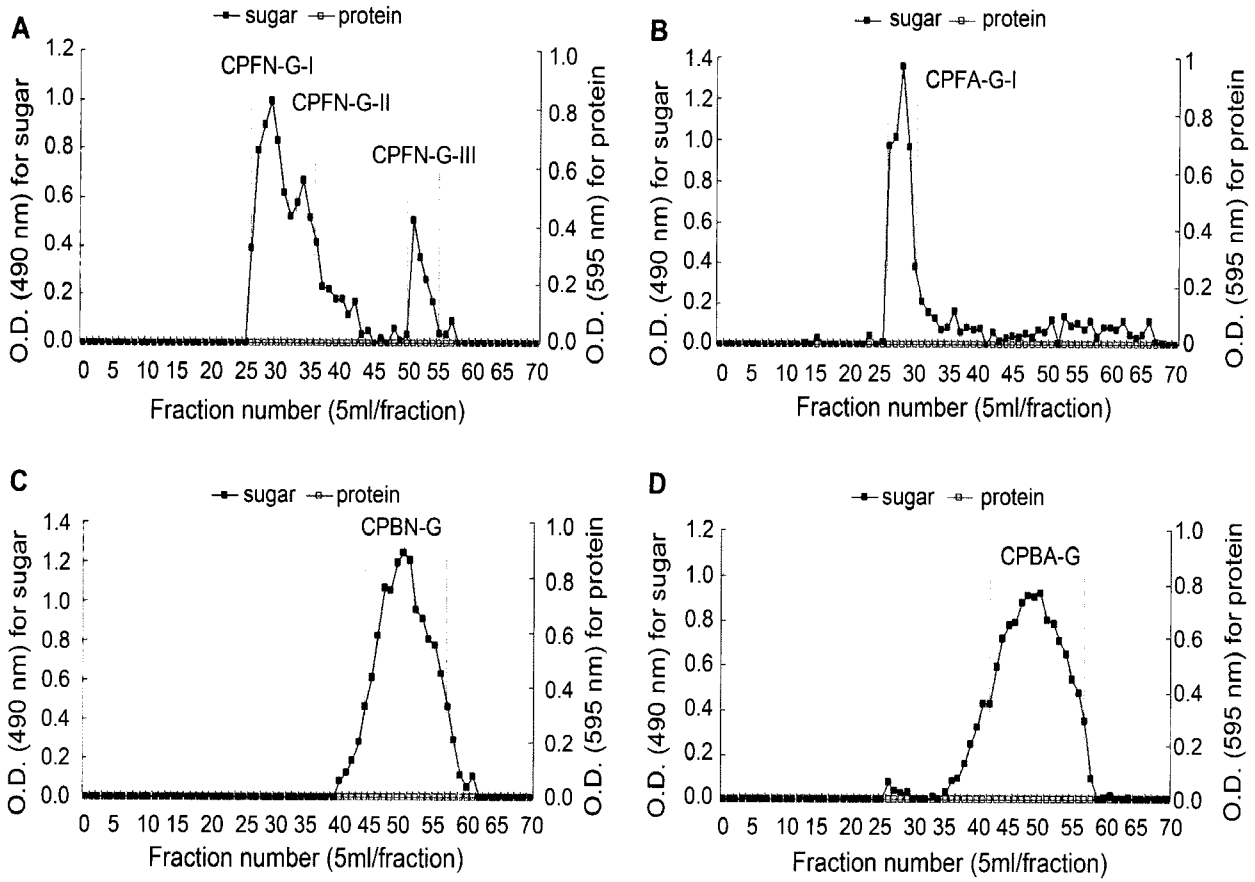

Fig. 2. Gel filtration chromatograms of each acidic and neutral polysaccharide fraction on a Sepharose CL-6B column (2.4 × 80 cm).
A. Chromatogram of the neutral polysaccharide fraction extracted from the fruiting body (fraction numbers of ion-exchange chromatography: 14–19). **B**. Chromatogram of the acidic polysaccharide fraction extracted from the fruiting body (fraction numbers of ion-exchange chromatography: 45–53). **C**. Chromatogram of the neutral polysaccharide fraction extracted from the cultured cell-free broth (fraction numbers of ion-exchange chromatography: 13–17). **D**. Chromatogram of the acidic polysaccharide fraction extracted from the cultured cell-free broth (fraction numbers of ion-exchange chromatography: 47–55).

reported previously [9]. The nitrite in the culture supernatants was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μl samples.

TNF-α Production

The induction effect of each polysaccharide purified from *L. edodes*, which was solubilized in culture medium, on TNF-α production from LPS-treated RAW264.7 cells was determined as described previously [9]. Supernatants were harvested and the concentration of TNF-α was determined using ELISA kits (Biosource International, Camarillo, U.S.A.) according to the manufacturer's instructions.

Statistical Analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of the differences between the values determined for the various experimental and control groups. Data are expressed as means±standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *P* values of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

Exopolysaccharides Production by Fermentation

In the shaken flask culture, the composition of the medium used for polysaccharide production was as follows; glucose 60 g/l, yeast extract 10 g/l, KH_2PO4 2.0 g/l, and $MgSO_4 \cdot 7H_2O$ 1.0 g/l. In the bioreactor, the agitation and aeration levels were varied in order to maximize polysaccharide production. From these experiments, a maximum polysaccharide production of 7.34 g/l at 7 days was achieved using 300 rpm and 1.5 vvm in a previous work [26]. These optimized conditions resulted in a higher polysaccharide production in less days than what was previously reported [25, 35].

Purification, Fractionation, and Molecular Mass

The first stage of purification and fractionation, ion-exchange chromatography through DEAE-cellulose columns (2.5×50 cm), separated neutral polysaccharides from acidic fractions (Fig. 1). The yields of CPFN and CPFA obtained from CPF, which were the polysaccharides extracted using ethanol precipitation and dialysis after hot-water extraction of the fruiting body, were 32.1 mg/100 mg and 40.4 mg/100 mg, respectively (Fig. 1A). The yield of CPBN obtained from CPB, which was the polysaccharide extracted using ethanol precipitation and dialysis of the culture cell-free broth, was 28.7 mg/100 mg, and the yield of CPBA, which constituted the acidic fraction was 47.3 mg/100 mg (Fig. 1B). The crude polysaccharides from the fruiting body and culture broth both formed a light-brown powder, and the neutral and acidic polysaccharides formed a white powder. This indicates that the colored parts were separated from the black parts during ion-exchange chromatography, which has a high NaCl density [44].

The molecular mass distribution of each neutral and acidic fraction was investigated using gel filtration chromatography with a Sepharose CL-6B column (2.4×80 cm). This technique resulted in six polysaccharide fractions, namely CPFN-G-I, CPFN-G-II, CPFN-G-III, CPFA-G, CPBN-G, and CPBA-G (Fig. 2), all of which contained no protein. This most likely occurred because all non-bound proteins were removed during ion-exchange chromatography and dialysis. The molecular masses of the six polysaccharide fractions ranged from 31 to 658 kDa, as determined by gel filtration chromatography when dextran was used as the standard (Fig. 3). Lentinan, which was the polysaccharide from the fruiting body, was found to have a molecular mass ranging from 400 to 800 kDa. It has been previously reported that polysaccharides extracted from the fruiting body by hot-water extraction had a molecular mass of 200 kDa [46]. In this study, the fraction was obtained not only within the same range but also within a smaller range than what was reported in the fractions of the fruiting body and culture broth.

Macrophage Activation by Polysaccharides

The cytotoxicity of each polysaccharide fraction was tested in the macrophage-like cell line, RAW264.7 cells. From this analysis, treatment with each polysaccharide was found not to appreciably affect cell viability (Fig. 4A). Since macrophages act as regulatory and effector cells in the immune system, understanding the activation of macrophages is expected to aid in the development of therapeutics to treat microbial pathogens and cancers [18, 31].

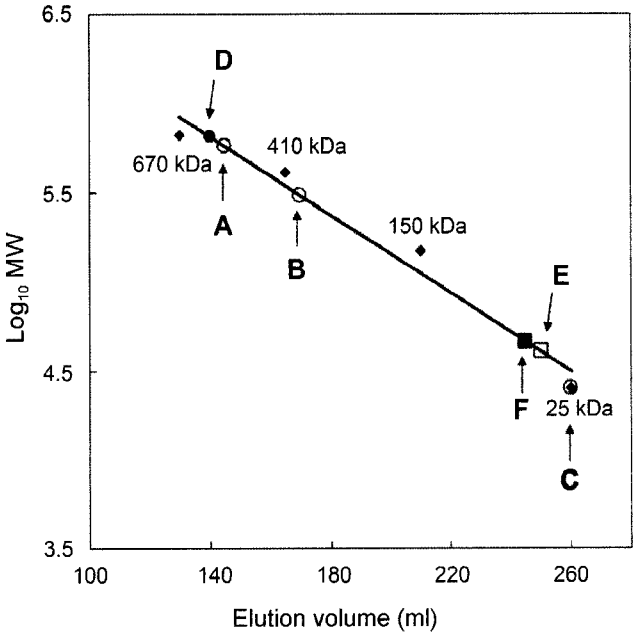

Fig. 3. Average molecular mass of each purified polysaccharide extracted from the cultured cell-free broth and the fruiting body by Sepharose CL-6B gel filtration chromatography.
A. CPFN-G-I, 580 kDa. **B.** CPFN-G-II, 308 kDa. **C.** CPFN-G-III, 25 kDa. **D.** CPFA-G, 658 kDa. **E.** CPBN-G, 40 kDa. **F.** CPBA-G, 46 kDa.

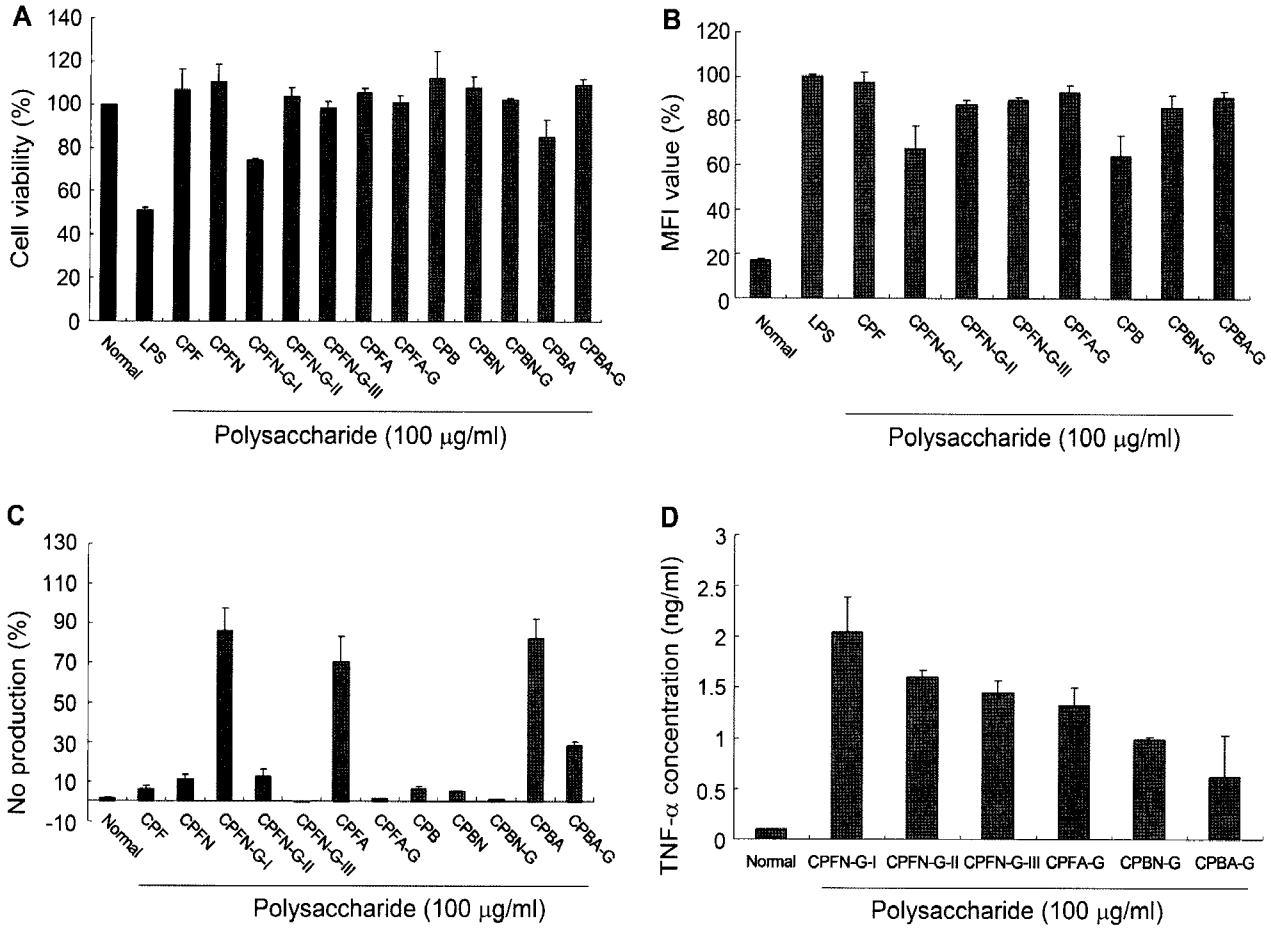

Fig. 4. Immunostimulating effects of purified polysaccharides extracted from the cultured cell-free broth and the fruiting body by DEAE cellulose and Sepharose CL-6B.

A. The effects of polysaccharides from the cultured cell-free broth and the fruiting body on macrophage cell viability. **B**. The effects of purified polysaccharides extracted from the cultured cell-free broth and the fruiting body on the macrophage phagocytosis. The mean fluorescence after drug treatment was compared with the control and represents the phagocytosis index. **C**. The effects of isolated and purified polysaccharides from the cultured cell-free broth and the fruiting body on NO synthesis in murine macrophage-like cell. **D**. The effects of purified polysaccharides from the cultured cell-free broth and the fruiting body on TNF-α production in RAW264.7 cells. Each column represents the mean±SEM of at least three independent experiments.

Phagocytosis is the first step in the macrophages' response to pathogens. In this process, macrophages will process and present antigens to the lymphocytes by engulfing and digesting the invading pathogens, which in turn elevates the innate immune response [41]. To determine the effects of each polysaccharide fraction on the phagocytic activity of macrophages, the uptake of FITC-labeled dextran in polysaccharide treated and untreated macrophages was compared (Fig. 4B). From this analysis, we found that all the polysaccharides increased the phagocytotic uptake of the RAW264.7 cells. In addition, macrophages can defend against pathogen invasion by secreting cytokines, such as the tumor necrosis factor-α (TNF-α), and inflammation mediators such as NO [29]. The role of NO in the anticancer effect of rodent macrophages has been well established [11, 13, 20]. Macrophages were incubated with 100 μg/ml of each polysaccharide and the amount of NO production was measured and compared with that produced by the untreated control group (Fig. 4C). The amount of NO production in all the polysaccharides-treated cells was higher than the untreated control RAW264.7 cells, where CPFN-G-III produced the most NO. These results are in agreement with a previous study, where polysaccharides from Basidiomycete mushrooms were shown to facilitate the release of NO by activating macrophages [17, 19, 22, 27]. TNF-α, which is also produced by macrophages, is considered the most important mediator directly involved in killing tumor cells [38]. After TNF binds to its receptor, the complex becomes internalized by endocytosis and then follows multiple diverse intracellular pathways. It has been reported that TNF affects the production of reactive oxygen species in the mitochondria, which results in plasma membrane permeabilization, iNOS expression induction, breaks in DNA strands, and induction of serine protease activity [1, 14, 33, 40]. Ultimately, these processes are involved in cell death [30]. The amounts of TNF-α produced in RAW264.7 cells after treatment with 100 μg/ml of each polysaccharide were higher than the amount produced in

untreated cells. Among all the polysaccharides, CPFN-G-I, which was the polysaccharide from the fruiting body, showed the highest productivity (Fig. 4D).

Acknowledgment

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

REFERENCES

1. Baloch, Z., S. Cohen, and F. D. Coffman. Synergistic interactions between tumor necrosis factor and inhibitors of DNA topoisomerase I and II. *J. Immunol.* **145:** 2908–2913.
2. Borchers, A. T., J. S. Stern, R. M. Hackman, C. L. Keen, and M. E. Gershwin. 1999. Mushrooms, tumors, and immunity. *Proc. Soc. Exp. Biol. Med.* **221:** 281–293.
3. Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
4. Bruneteau, M., I. Fabre, J. Perret, G. Michel, P. Ricci, J. P. Joseleau, *et al.* 1988. Antitumor active β-D-glucans from *Phytophthora parasitica*. *Carbohydr. Res.* **175:** 137–143.
5. Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **47:** 936–942.
6. Chaplin, M. F. and J. F. Kennedy. 1986. *Carbohydrate Chemistry.* Oxford IRL Press.
7. Chihara, G., J. Hamuro, Y. Maeda, Y. Arai, and F. Fukuoka. 1970. Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **30:** 2776–2781.
8. Chihara, G., Y. Maeda, J. Hamuro, T. Sasaki, and F. Fumiko. 1969. Inhibition of mouse sarcoma 180 by polysaccharides from *Lentinus edodes*. *Nature* **222:** 687–688.
9. Cho, J. Y., K. U. Baik, J. H. Jung, and M. H. Park. 2000. *In vitro* anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. *Eur. J. Pharmacol.* **398:** 399–407.
10. Collins, L., T. Zhu, J. Guo, Z. J. Xiao, and C. Y. Chen. 2006. *Phellinus linteus* sensitizes apoptosis induced by doxorubicin in prostate cancer. *Br. J. Cancer* **95:** 282–288.
11. Cui, S., J. S. Reichner, R. B. Mateo, and J. E. Albina. 1994. Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res.* **54:** 2462–2467.
12. Duperrier, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Yoneda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated *in vitro* from monocytes, in the presence of different serum supplements. *J. Immunol. Methods* **238:** 119–131.
13. Farias Eisner, R., M. P. Sherman, E. Aeberhard, and G. Chaudhuri. 1994. Nitric oxide is an important mediator for tumoricidal activity *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 9407–9411.
14. Goossens, V., J. Grooten, K. De Vos, and W. Fiers. 1995. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 8115–8119.
15. Gregory, F. J., E. M. Healy, H. P. Agersborg Jr., and G. H. Warren. 1966. Studies on antitumor substances produced by Basidiomycetes. *Mycologia* **58:** 80–90.
16. Griffith, T. S., S. R. Wiley, M. Z. Kubin, L. M. Sedger, C. R. Maliszewski, and N. A. Fanger. 1999. Monocyte-mediated tumoricidal activity *via* the tumor necrosis factor-related cytokine. *J. Exp. Med.* **189:** 1343–1354.
17. Han, M. D., E. S. Lee, Y. K. Kim, J. W. Lee, H. Jeong, and K. H. Yoon. 1998. Production of nitric oxide in RAW264.7 macrophages treated with ganoderan, the β-glucan of *Ganoderma lucidum*. *Kor. J. Mycol.* **26:** 246–255.
18. Hibbs Jr., J. B. 1976. The macrophage as a tumoricidal effector cell: A review of *in vivo* and *in vitro* studies on the mechanism of the activator macrophage nonspecific cytotoxic reactions. *In: The Macrophage in Neoplasia.* Academic Press, New York, pp. 83–91.
19. Jung, Y. S., B. K. Yang, Y. T. Jeong, R. Islam, S. M. Kim, and C. H. Song. 2008. Immunomodulating activities of water-soluble exopolysaccharides obtained from submerged culture of *Lentinus lepideus*. *J. Microbiol. Biotechnol.* **18:** 1431–1438.
20. Keller, R., M. Geiges, and R. Keist. 1990. L-Arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res.* **50:** 1421–1425.
21. Kiener, P. A., P. M. Davis, B. M. Rankin, S. J. Klebanoff, J. A. Ledbetter, G. C. Starling, and W. C. Liles. 1997. Human monocytic cells contain high levels of intracellular Fas ligand: Rapid release following cellular activation. *J. Immunol.* **159:** 1594–1598.
22. Kim, J. Y., S. E. Byeon, Y. G. Lee, Y. J. Lee, J. S. Park, E. K. Hong, and J. Y. Cho. 2008. Immunostimulatory activities of polysaccharides from liquid culture of pine-mushroom *Tricholoma matsutake*. *J. Microbiol. Biotechnol.* **18:** 95–103.
23. Komatsu, N., S. Okubo, S. Kikumoto, K. Kimura, and G. Saito. 1969. Host-mediated antitumor action of schizophyllan, a glucan produced by *Schizophyllum commune*. *Gann* **60:** 137–144.
24. Kroncke, K. D., K. Fehsel, and V. Kolb-Bachofen. 1995. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol. Chem. Hoppe Seyler* **376:** 327–343.
25. Lee, B. W., G. H. Im, D. W. Kim, K. M. Park, S. H. Son, and T. H. Shon. 1993. Cultural characteristics and pilot scale fermentation for the submerged mycelial culture of *Lentinus edodes*. *Kor. J. Appl. Microbiol. Biotechnol.* **21:** 609–614.
26. Lee, H. H., J. Y. Cho, and E. K. Hong. 2007. Exo-polysaccharide production from liquid culture of *Lentinus edodes*. *Kor. J. Biotechnol. Bioeng.* **22:** 1–6.
27. Lorenzen, K. and T. Anke. 1998. Biologically active metabolites from basidiomycetes. *Curr. Org. Chem.* **2:** 329–364.
28. Mackay, R. J. and S. W. Pussell. 1986. Protein changes associated with stages of activation of mouse macrophages for tumor cell killing. *J. Immunol.* **137:** 1392–1398.
29. MacMicking, J., Q. W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15:** 323–350.

30. Monastra, G., A. Cabrelle, A. Zambon, A. Rosato, B. Macino, D. Collavo, and P. Zanovello. 1996. Membrane form of TNF alpha induces both cell lysis and apoptosis in susceptible target cells. *Cell Immunol.* **171:** 102–110.
31. Nathan, C. F., S. C. Silrerstein, L. D. Bruker, and Z. A. Lohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. *J. Exp. Med.* **149:** 100–113.
32. Ng, T. B. and H. X. Wang. 2005. Pharmacological actions of *Cordyceps*, a prized folk medicine. *J. Pharm. Pharmacol.* **57:** 1509–1520.
33. Ogura, T., M. Tatemichi, and H. Esumi. 1997. TNF-alpha mediates inducible nitric oxide synthase expression in human neuroblastoma cell line by cisplatin. *Biochem. Biophys. Res. Commun.* **233:** 788–791.
34. Ohno, N., Y. Adachi, I. Suzuki, K. Sato, S. Oikawa, and T. Yadomae. 1986. Characterization of the antitumor glucan obtained from liquid-cultured *Grifola frondosa*. *Chem. Pharm. Bull.* **34:** 1709–1715.
35. Park, K. S. and B. N. Lee. 1997. Extraction and separation of protein-bound polysaccharide by *Lentinus edodes*. *Kor. J. Food Nutr.* **10:** 503–508.
36. Roland, J. F., Z. F. Chmiolewicz, B. A. Weiner, A. M. Gross, O. P. Boening, J. V. Luck, *et al.* 1960. Calvacin: A new antitumor agent. *Science* **132:** 1987.
37. Sasaki, T., N. Takasuka, G. Chihara, and Y. Y. Maeda. 1976. Antitumor activity of degraded products of lentinan: Its correlation with molecular weight. *Gann* **67:** 191–195.
38. Shu, C. H., B. J. Wen, and K. J. Lin. 2003. Monitoring the polysaccharide quality of *Agaricus blazei* in submerged culture by examining molecular weight distribution and TNF-α release capability of macrophage cell line RAW264.7. *Biotechnol. Lett.* **25:** 2061–2064.
39. Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* **248:** 1019–1023.
40. Suffys, P., R. Beyaert, F. Van Roy, and W. Fiers. 1988. Involvement of a serine protease in tumour-necrosis-factor-mediated cytotoxicity. *Eur. J. Biochem.* **178:** 257–265.
41. Takahashi, K., M. Yamazaki, and S. Abe. 1988. Local induction of a tumor necrosis factor (TNF)-like cytotoxic factor in murine tissues with tumorous and nontumorous inflammation after systemic administration of antitumor polysaccharides. *J. Pharmacobiodynam.* **11:** 472–478.
42. Tsukagoshi, S. and F. Ophashi. 1974. Protein-bound polysaccharide preparation, PS-K, effective against mouse sarcoma-180 and rat ascites hepatoma AH-13 by oral use. *Gann* **65:** 557–578.
43. Ukai, S., T. Kiho, C. Hara, M. Morita, A. Goto, N. Imazumi, and Y. Hasegawa. 1983. Polysaccharides in fungi. XIII. Antitumor activity of various polysaccharides isolated from *Dictyophora indusiata*, *Ganoderma japonicum*, *Cordyceps cicadae*, *Auricularia auricular-judae*, and *Auricularia* species. *Chem. Pharm. Bull.* **31:** 741–744.
44. Ukawa, Y., H. Ito, and M. Hisamatsu. 2000. Antitumor effects of (1→3)-β-D-glucan and (1→6)-β-D-glucan purified from newly cultivated mushroom, Hatakeshimeji (*Lyophyllum decastes* Sing.). *J. Biosci. Bioeng.* **90:** 98–104.
45. Wasser, P. D. 2002. Medicinal mushroom as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotech.* **60:** 258–274.
46. Zheng, R., S. Jie, D. Hanchuan, and W. Moucheng. 2005. Characterization and immunomodulating activities of polysaccharide from *Lentinus edodes*. *Int. Immunopharmacol.* **5:** 811–820.