Available online at www.sciencedirect.com
SCIENCE DIRECT®
Life Sciences 79 (2006) 72–80
Life Sciences
www.elsevier.com/locate/lifescie

Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*

Yong Ook Kim [a], Hae Woong Park [a], Jong Hoon Kim [a], Jae Young Lee [a], Seong Hoon Moon [b], Chul Soo Shin [a,*]

[a] *Department of Biotechnology, College of Engineering, Yonsei University, Shinchon-dong, Seodaemoon-gu, Seoul 120-749, South Korea*
[b] *Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Oun-dong, Yusung-gu, Daejon 305-333, South Korea*

Received 4 October 2005; accepted 14 December 2005

Abstract

The endo-polysaccharide extracted from mycelia of *Inonotus obliquus* (Pers.:Fr.) Pil. (Hymenochaetaceae) is a specific activator of B cells and macrophages. However, the in vivo anti-cancer effects and the chemical structure of the endo-polysaccharide are unknown. We purified the endo-polysaccharide, investigated its anti-cancer effects via in vitro and in vivo assays, and performed a structural characterization. The endo-polysaccharide was extracted from *I. obliquus* mycelia cultivated in a 300-l pilot fermenter, followed by hot water extraction and ethanol precipitation. Purification was achieved by DEAE-cellulose ion-exchange chromatography and gel-permeation chromatography. Chemical analysis revealed that the purified endo-polysaccharide is an α-linked fucoglucomannan with a molecular weight of approximately 1000 kDa. The anti-cancer activities of the endo-polysaccharide against various types of tumor cells were determined. No direct toxicity against either cancer or normal cells was observed. Intraperitoneal administration of the endo-polysaccharide significantly prolonged the survival rate of B16F10-implanted mice, resulting in a 4.07-fold increase in the survival rate at a dose of 30 mg/kg/day. After 60 days of feeding, approximately 67% of the initial number of mice survived with no tumor incidence based on macroscopic examination. These results indicate that the anti-cancer effect of endo-polysaccharide is not directly tumorcidal but rather is immuno-stimulating.

Keywords: *Inonotus obliquus*; Endo-polysaccharide; Anti-cancer activity; Immuno-stimulating; Chemical structure

Introduction

Inonotus obliquus (Pers.:Fr.) Pil. (syn; *Fuscoporia obliqua* (Pers.:Fr.) Aoshima) is a white rot fungus that belongs to the family Hymenochaetaceae of Basidiomycetes. This fungus is usually found as a sterile conk (sclerotia) called 'Chaga' on *Betula* species in nature (Campbell and Davidson, 1938). Since the sixteenth century, 'Chaga' has been used as a folk remedy in Russia and western Siberia. A decoction of fungal sclerotia did not show toxic effects and has been used in treatment of cancers and digestive system diseases (Saar, 1991). In recent years, many polyphenolic compounds, triterpenoids, and steroids, such as lanosterol, inotodiol, trametenolic acids, and ergosterol peroxides from *Inonotus* sclerotia, have shown various biological activities, including hypoglycemic (Mizuno et al., 1999), anti-viral (Ichimura et al., 1999), anti-fungal (Kahlos, 1994), hepato-protective (Solomon and Alexander, 1999), and anti-tumor (Kahlos et al., 1987; Jarosz et al., 1990; Mizuno, 1999) effects. Recently, there have been reports (Cui et al., 2005) that melanin complexes from sclerotia contain strong anti-oxidants and cyto-protectives.

Sclerotia extracts of *I. obliquus* are known to inhibit the growth and protein synthesis of tumor cells. However, Mizuno et al. (1999) reported that polysaccharides from fungal sclerotia, which are known to include hetero-polysaccharide and homo-glycan, showed strong anti-tumor effects while polysaccharides from cultured mycelia did not.

In a previous study, we reported that the endo-polysaccharide produced by a submerged culture of *I. obliquus* strongly stimulated humoral immunity related to B lymphocytes and

* Corresponding author. Tel.: +82 2 2123 2886; fax: +82 2 362 7265.
E-mail address: csshin@yonsei.ac.kr (C.S. Shin).

0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2005.12.047

macrophages (Kim et al., 2005). However, there is no information about the in vivo anti-tumor effect of endo-polysaccharide from *I. obliquus* mycelia. Therefore, we extracted and purified endo-polysaccharide from a submerged culture of *I. obliquus*. The in vitro and in vivo anti-tumor activities of the endo-polysaccharide were investigated and the chemical structure was characterized.

Materials and methods

Preparation of endo-polysaccharide

Strain and cultivation method

I. obliquus BELYU1102 (Kim et al., 2005) isolated from the sclerotium of *Betula platyphylla* collected in Hokkaido (Japan) was used in this study. The fungus was incubated on YMA (Difco Lab., USA) slants at 27 °C for 7 days then kept at 4 °C for stock. Endo-polysaccharide was extracted from cultivated mycelia following the previously described method of Kim et al. (2005). Briefly, a modified YM broth was used as both a seed and a fermentation medium. Seed cultivations were carried out in 500 ml baffled flasks for 100 h on a shaking incubator at 27 °C and 140 rpm. Six hundred milliliters of the culture broth was added to a 30-l fermenter containing 19.4 l of fresh YM medium. After 186 h of cultivation, 16.5 l of the culture broth was added as an inoculum to a 300-l fermenter (stirred type; Kobio Tech., Seoul, Korea) containing 165 l of the modified YM medium. The 300-l fermenter was equipped with four equally spaced Rushton disk turbines with six flat blades. The pH was initially set at 6.0 with either 1 N HCl or NH_4OH, and thereafter was not controlled. The dissolved oxygen tension (DOT) was initially set at a 100% saturation level and was maintained at 20% or higher during cultivation by controlling the air flow (0.5–1.0 vvm) and agitation rates (70–150 rpm). Cultivations were then performed for 305 h. The internal pressure was maintained in the range of 0.1–0.25 kg/cm^3.

Mycelia, obtained by filtration of culture broths for isolation of water-soluble endo-polysaccharide were washed 3 times with distilled water, then suspended in distilled water. The solutions were heated for 6 h in an autoclave at 121 °C to extract heat-stable endo-polysaccharide from mycelia, followed by filtration. The filtrates were then put through a process of ethanol precipitation, dialysis, and lyophilization. The lyophilized samples were dissolved in distilled water then water-soluble endo-polysaccharide was isolated by centrifugation at for 60 min at 10,447×*g* (Fig. 1).

Purification of endo-polysaccharide

Three grams of water-soluble endo-polysaccharide was applied to a DEAE-cellulose column (Merck, Art 3201, 4×35 cm) that had been equilibrated with 5 mM sodium phosphate buffer (pH 7.8) (Fig. 1). After the column was washed with the same buffer (total of 1000 ml), it was eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer (total of 2000 ml). Fractions were collected using an elution pattern, then concentrated in an evaporator at 60 °C. The concentrate was dialyzed in distilled water for 72 h, followed by freeze-drying. For gel permeation chromatography, samples were dissolved in 20 ml of buffer, then applied to a gel permeation column (Toyopearl HW65F (1.5×120 mm); Tosoh Co., Ltd., Japan), and eluted at 0.5 ml/min with 5 mM sodium phosphate buffer (pH 7.8) (Fig. 1). The fractions were collected using an elution pattern and concentrated in an evaporator at 60 °C. The concentrate was dialyzed in distilled water for 72 h, then freeze-dried.

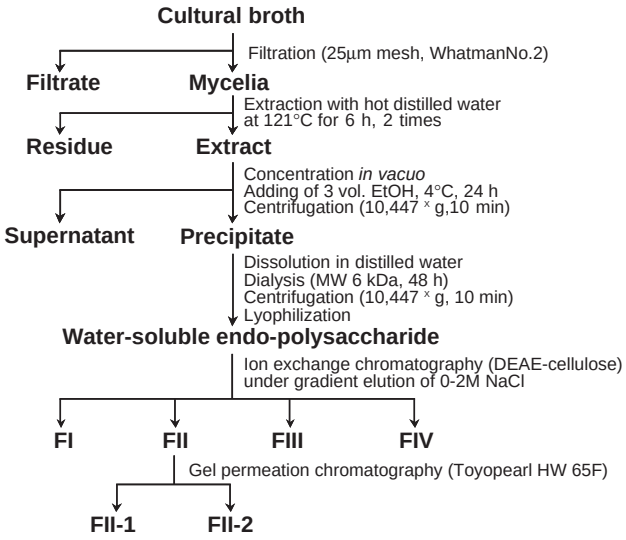

Fig. 1. Purification scheme of the endo-polysaccharide with immuno-stimulating anti-cancer activity from cultured mycelia of *I. obliquus*.

Preparation of mice, cell lines, and media

Specific pathogen-free (SPF) BDF1 mice (female, 4–6 weeks old) were purchased from Charles River Korea (Orient Inc., Korea). They were acclimatized under controlled conditions for 1 week before experimental feeding with a commercial rat feed (Orient Inc). Sterilized food and water were supplied ad libitum.

To investigate in vitro direct cytotoxicity of endo-polysaccharide various cancer cell lines of Hur7 (human hepatoma), HEC-1B (human endometrial epithelial cells), B16F10 (murine melanoma), A549 (human lung carcinoma), KATO-III (human stomach carcinoma), SW156 (kidney adenocarcinoma), MCF-7 (human breast adenocarcinoma), and SK-OV3 (human ovary adenocarcinoma) were used. Normal cell lines of RAW 264.7 (murine macrophage), HUVEC (human umbilical vein endothelial cells), and HEK293T (human embryo kidney cells) were also used. Cells were purchased from the American Type Culture Collection (ATCC; USA). RAW 264.7 cells were used for immuno-activity-guided fractionation. RAW 264.7, HEK293T, Hur7, and MCF-7 cells were maintained in DMEM, while HUVEC, HEC-1B, B16F10, A549, KATO-III, SW156, and SK-OV3 cells were kept in RPMI-1640. All media were purchased from Gibco BRL (NY, USA), and supplemented with 10% fetal bovine serum (HyClone, USA), 100 U/ml of penicillin, and 100 g/ml of streptomycin. The cells were

incubated in a 5% CO_2 incubator at 37 °C and maintained in designated media. The culture broths were substituted with fresh medium every 2 or 3 days.

Measurement of the nitric oxide producing activity of macrophages

The immuno-stimulating activity of fractionated endo-polysaccharide samples was determined by nitrite levels produced by RAW 264.7 macrophages. Approximately 5×10^5 cells/ml of macrophage cells were placed on 24-well plates containing either endo-polysaccharide or lipopolysaccharide (LPS; from *Salmonella typhosa*) then incubated for 24 h at 37 °C. Supernatants were obtained and mixed with an equal volume of Griess reagent. The amount of evolved nitrite was determined by measuring the absorbance at 540 nm (Kim et al., 2005). Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, USA).

In vivo experiments using mice

The inhibitory effect of endo-polysaccharide against a murine tumor was investigated. BDF1 mice were randomly divided into nine groups consisting of eight or nine per group. After the mice were intraperitoneally implanted with 0.2 ml of 1×10^5 B16F10 melanoma cells per mouse on day 0, endo-polysaccharide was supplied daily intraperitoneally at a dose of 3, 10, or 30 mg/kg/day or orally at a dose of 30, 100, or 300 mg/kg/day until all vehicle mice died (Han et al., 1999). Vehicle mice treated with equal amounts of a 0.85% NaCl solution or 3rd distilled water were used as a control. The body weight of mice was measured every day during the treatment period in order to detect endo-polysaccharide toxicity. The number of surviving mice was counted every day for 60 days. After each mouse died, a postmortem examination was performed to check for tumors. The result was expressed as Survival rate (%)=(MST of treated group/MST of control group)×100 (MST: mean survival time).

Cytotoxicity test

A direct endo-polysaccharide cytotoxicity test was performed. After each cancer cell line was adjusted to a level of 1×10^5 cells/ml, 100 μl of the cell suspension was put in each well of a replicate 96-well plate. Then, 100 μl of various polysaccharide solutions (10, 50, 100, and 200 μg/ml) was added to the wells. After 48 h of incubation, an MTT assay was done (Pagè et al., 1988). Fifty microliters of MTT (3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml; Sigma, USA) was added to each well of a plate then incubated for 4 h at 37 °C. The formazan crystals that formed were dissolved in 100 μl of DMSO followed by an optical density measurement at 540 nm using a micro-plate reader. The cytotoxicity was calculated as Cytotoxicity (%)=[(Total cell number−Viable cell number)/Total cell number]×100.

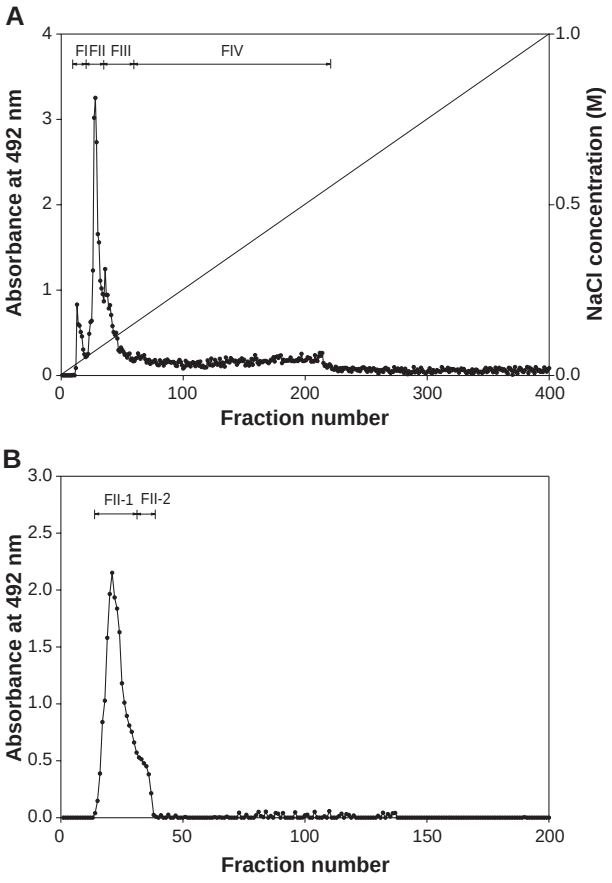

Fig. 2. Elution profiles for ion-exchange chromatography of water-soluble endo-polysaccharide (A), and gel permeation chromatography of FII (B). The straight line in (A) indicates the NaCl gradient. ●: Total sugar content.

Procedures for chemical analysis of endo-polysaccharide

NMR spectroscopy

^{13}C and ^{1}H NMR spectroscopic analyses were performed using a Bruker Advance 600 and a Bruker Advance DPX-300, respectively. Trace amounts of water in the purified polysaccharide were eliminated using pure D_2O (Aldrich, USA), followed by freeze-drying. This procedure was repeated three times.

Determination of the sugar content and molecular weight

The total sugar and protein contents of endo-polysaccharide were determined, respectively, by the phenol-sulfuric acid method and the modified Bradford method (Kim et al., 2005). The sugar composition of endo-polysaccharide was also analyzed (Korea Basic Science Institute, Daejeon, Korea). An amount of 10 μl of 1% purified endo-polysaccharide was hydrolyzed with either 2 M trifluoroacetic acid (TFA) or 6 N HCl for 4 h at 100 °C, followed by centrifugation for 30 min at 32,600×*g* and syringe filtration (0.2 μm pore size). The solutions were then evaporated to remove the remaining acid. Ten microliters of hydrolyzed solution was injected into a high pH anion exchange chromatograph (HPAEC-PAD) consisting of a Bio-LC DX-600 chromatograph (Dionex, USA) equipped with a CarboPac PA1 column (2.0×250 mm; Dionex, USA) and

Table 1
Sugar compositions of the endo-polysaccharide fractions obtained from ion exchange chromatography and gel permeation chromatography

Sugar	Fractions				
	FI (%)	FII-1 (%)	FII-2 (%)	FIII (%)	FIV (%)
Mannose	45.1	70.8	74.4	41.5	23.5
Glucose	5.2	1.6	2.6	2.4	15.3
Fucose	0.6	0.8	0.7	0.2	0.3
Galactose					1.6
Glucosamine		0.1	0.1		
Galactosamine					
Total sugar content	50.9	73.2	77.9	44.0	40.7
Total protein content	49.1	26.8	22.1	56.0	59.3

FI, FII, FIII, and FIV were purified through ion exchange chromatography, and their neutral sugar contents were analyzed by HPAEC-PAD. FII-1 and FII-2 were purified by gel permeation chromatography, and their neutral and amino sugar contents were analyzed by HPAEC-PAD.

an integrated amerometric ED50 detector. The sugar content was then determined.

For molecular mass determination of the endo-polysaccharide, high performance gel permeation chromatography was performed on an LC-10 chromatograph equipped with a Shodex OHpak SB-804 column (MW: $\sim 10^6$, 8.0×300 mm; Showa, Japan) and an RI detector (Polymer Lab. Ltd., Japan). Thirty microliters of the 1% purified endo-polysaccharide fraction was injected and eluted at a water flow rate of 1 ml/min at room temperature. Various molecular weights of dextrans (MW: 188,000, 150,000, 75,000, 40,000, 17,500, and 10,000 Da; Sigma Chem. Co., USA) were used as standards.

Results

Purification of endo-polysaccharide and sugar content determination

Water-soluble endo-polysaccharide was obtained by mycelial cultivation, water extraction, and ethanol precipitation. The lyophilized polysaccharide was subjected to DEAE-cellulose ion exchange chromatography with NaCl elution resulting in FI, FII, FIII, and FIV peaks (30.0, 154.1, 58.8, and 19.4 mg, respectively) (Fig. 2A). The FI, FII, and FIII fractions had no color while the FIV fraction was light yellow. The FII fraction's total sugar content was 75.0%, which was much higher than the other fractions (50.9%, 44.0%, and 40.7%, respectively, for FI, FII, and FIII). However, according to sugar analysis using HPAEC-PAD, the sugar compositions of the fractions were similar with mannose as the major component (Table 1).

The FII fraction (100 mg) was further separated into FII-1 and FII-2 (51.7 and 28.3 mg, respectively) by gel permeation chromatography (Fig. 2B). FI-1 and FII-2 were hetero-glycans consisting of mannose, glucose, fucose, and glucosamine whose corresponding percents (w/w) were 70.8:1.6:0.8:0.1 and 74.4:2.6:0.7:0.1, respectively. The molecular weight of FII-1 was estimated to be 1000 kDa by high performance gel

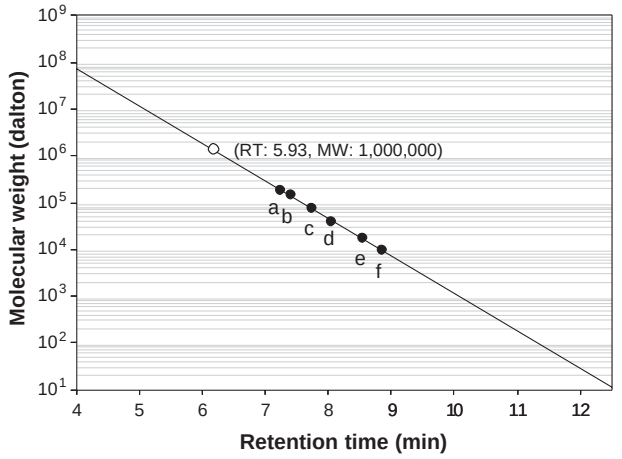

Fig. 3. Estimation of the molecular weight of endo-polysaccharide by gel-permeation HPLC with a GFC column. $R^2=0.998$. The standard compounds were dextrans with molecular weights of 1.88×10^5 (a), 1.5×10^5 (b), 7.5×10^4 (c), 4.0×10^4 (d), 1.75×10^4 (e), and 1.0×10^4 (f).

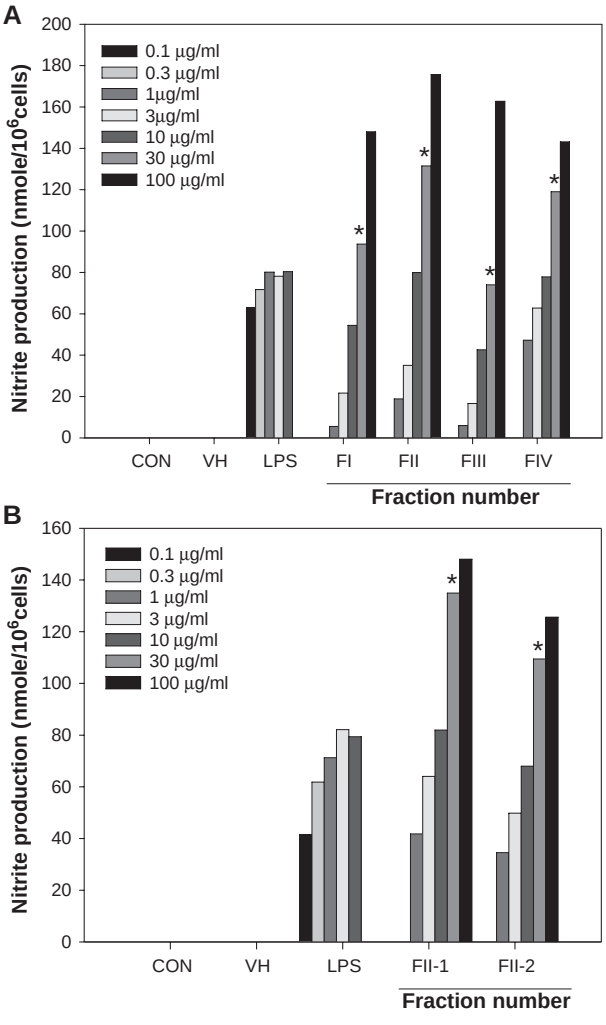

Fig. 4. The effect of endo-polysaccharide fractions separated through ion-exchange chromatography (A) and gel permeation chromatography (B) on the nitrite-producing activity of RAW264.7 cells. The LPS concentration was 0.1, 0.3, 1, 3, and 10 μg/ml, respectively, whereas the sample concentrations were 1, 3, 10, 30, and 100 μg/ml, respectively. Significance levels were determined using Student's *t*-test compared to a control group ($p<0.01$).

permeation chromatography (Fig. 3). FII-1 showed a homogeneous peak on HPGPC analysis.

The nitrite-producing activity of the four fractions was measured at a level of 1–100 μg/ml. There was no significant difference in the activities of the fractions (Fig. 4A). FII showed the highest activity and yield (Fig. 4A). Comparison of the activities of FII-1 and FII-2 obtained from additional purification showed that FII-1 had a higher activity (Fig. 4B). On the basis of this result, the most active fraction FII-1 was designated as the endo-polysaccharide to be used for further study.

Structural characterization of endo-polysaccharide

^{1}H and ^{13}C NMR analyses were performed for further analysis of endo-polysaccharide structure. Fractions FI, FII, and FIII showed similar patterns of ^{1}H and ^{13}C NMR results, but fraction FIV was different (data not shown). FII was further fractionated to FII-1 and FII-2 and both fractions showed similar patterns. The ^{1}H and ^{13}C NMR spectra of FII-1 are shown in Fig. 5. The ^{1}H NMR spectrum of FII-1 revealed nine anomeric proton signals at 5.47, 5.42, 5.25, 5.18, 5.16, 5.14, 5.09, 5.05, and 4.96 ppm. These data, along with the coupling constants of anomeric protons, indicate the existence of an α-linkage (Fig. 5A). The ^{13}C NMR spectrum showed an anomeric carbon at 102.41 ppm, and seven oxylated carbons were observed at 78.90, 76.22, 73.98, 73.04, 70.09, 66.66, and 61.42 ppm (Fig. 5B).

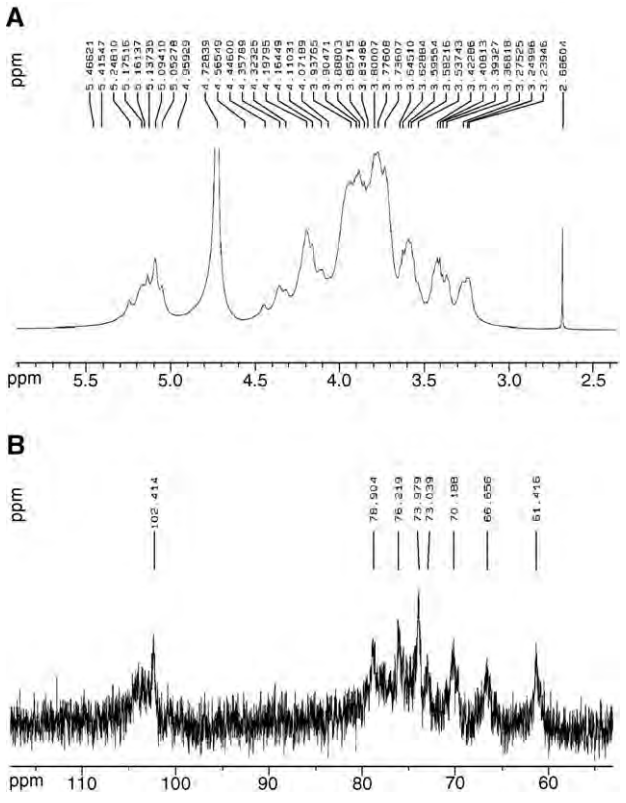

Fig. 5. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of the FII-1 endo-polysaccharide fraction obtained from gel permeation chromatography.

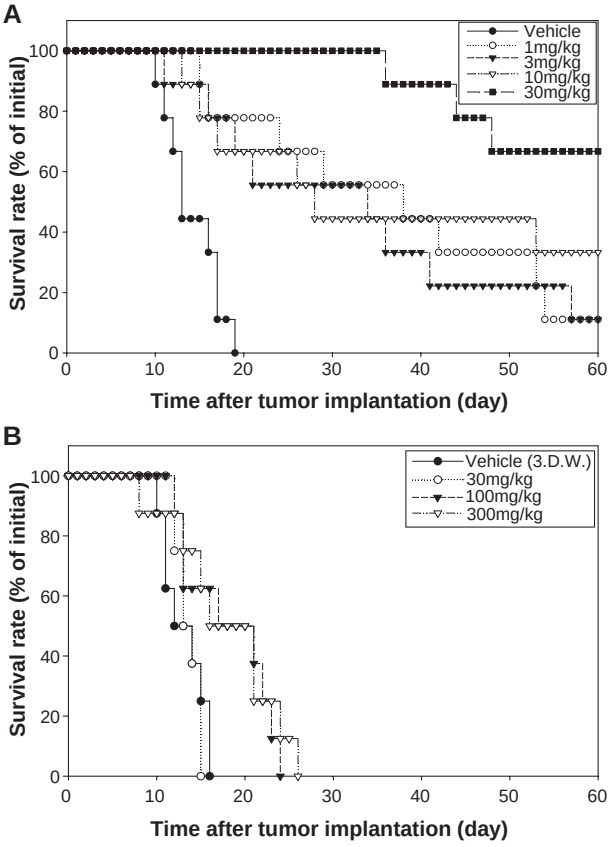

Fig. 6. Growth inhibition of B16F10 melanoma cells due to intraperitoneal (A) and oral (B) administration of endo-polysaccharides. Mice were intraperitoneally implanted with B16F10 melanoma cells on day 0. The endo-polysaccharide was administered intraperitoneally at 1, 3, 10, and 30 mg/kg, and orally at 30, 100, and 300 mg/kg from day 0 to day 9. Control group mice were fed with 0.85% NaCl (i.p.) or 3rd distilled water (oral). The number of animals per group was 9 ($n_{\text{i.p.}}=9$) or 8 ($n_{\text{oral}}=8$). The number of surviving animals was counted everyday for 60 days.

Effect of endo-polysaccharide on the survival rate of mice implanted with cancer cells

The immuno-stimulating and pharmacological activity of endo-polysaccharide was measured in vivo. After mice were inoculated with B16F10 melanoma cells, the effect of endo-polysaccharide on the survival rate was determined (Fig. 6). B16F10 melanoma cells were intraperitoneally implanted into mice on day 0. The mice were treated with intraperitoneal endo-polysaccharide for 9–10 days at 1, 3, 10 and 30 mg/kg/day, or fed orally at 30, 100, or 300 mg/kg/day until the first mouse of the untreated control group died. In the untreated group, the mice gained weight abnormally from the 5th day after inoculation of melanoma cells until death, probably due to rapid growth of melanoma cells in the peritoneal cavity. In all cases of endo-polysaccharide use, the survival rate of the mice was prolonged in a dose-dependent manner. The optimum feeding rates of intraperitoneal and oral administration were 30 mg/kg/day and 300 mg/kg/day, respectively. Intraperitoneal treatment (4.07-fold increase) was more effective in the survival rate than oral feeding (1.40-fold increase) (Table 2). The incidence of tumor tissue was not observed for 60-day survivors

Table 2
The effect of the endo-polysaccharide obtained from cultured mycelia of *Inonotus obliquus* on tumor growth

Treatment	Administration period	Dose (mg/kg)	Survival rate (%)	60 (30) day survivors
Vehicle (Saline)	Days 0–9	–	100.00	0 (0)
IP-treated	Days 0–9	1	271.43	1 (5)
IP-treated	Days 0–9	3	241.18	1 (5)
IP-treated	Days 0–9	10	273.95	3 (4)
IP-treated	Days 0–9	30	407.56	6 (9)
Vehicle (3.D.W.)	Days 0–9	–	100.00	–
Oral-treated	Days 0–9	30	104.12	–
Oral-treated	Days 0–9	100	141.24	–
Oral-treated	Days 0–9	300	140.21	–

The mean survival time of B16F10 melanoma-transplanted BDF1 mice was 14.5 (i.p.) and 15.2 (oral) day, respectively.

of intraperitoneal treatment groups at all (Fig. 7). Life-threatening toxic effect and body weight loss were not detected in any group of the mice administrated intraperitoneally and orally with endo-polysaccharide. As time passed, the body weight of intraperitoneally treated mice stopped increasing (Fig. 8A) while the weight of orally treated mice continued to increase (Fig. 8B). These results indicate that the endo-polysaccharide has a significant inhibitory effect on growth of melanoma tumor cells in BDF1 mice.

Cytotoxicity of endo-polysaccharide against cancer and normal cells

An MTT assay was performed with cancer and normal cell lines to clarify the endo-polysaccharide toxic effect against tumor cells. Fig. 9 shows that the viabilities of cancer and normal cells after 48 h of incubation with endo-polysaccharide were specific to the cell-type. Most cancer cells were not directly affected by the endo-polysaccharide treatment, even at a high concentration level (200 μg/ml),

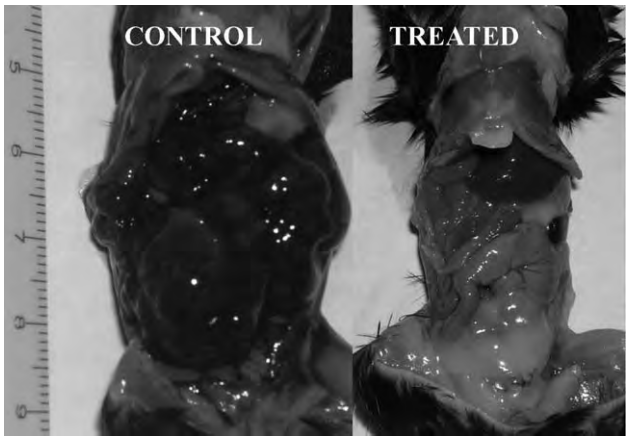

Fig. 7. Growth inhibition effect of endo-polysaccharide on melanoma solid tumor cells in vivo. BDF1 mice were implanted with B16F10 murine melanoma cells. They were treated intraperitoneally with either endo-polysaccharide (1, 3, 10, and 30 mg/kg per day) or 0.85% NaCl (vehicle) from day 0 to day 9 after tumor implantation. Surviving mice were dissected on day 60.

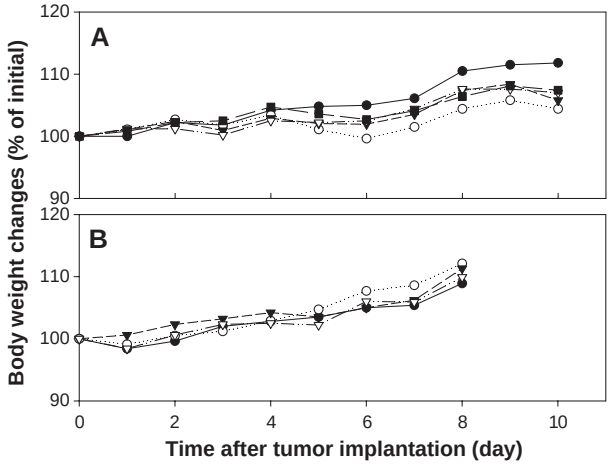

Fig. 8. Changes in body weight of B16F10 tumor-bearing mice. The body weights of the mice shown in Fig. 6A and B were measured. The mean body weight of mice prior to endo-polysaccharide treatment was 19.73±0.13 g (A), and 19.09±0.43 g (B), respectively. The body weight by day was expressed as a percent ratio to the initial body weight. (A) Vehicle (●); 1 mg/kg (○); 3 mg/kg (▼); 10 mg/kg (▽); 30 mg/kg (■). (B) Vehicle (●); 30 mg/kg (○); 100 mg/kg (▼); 300 mg/kg (▽).

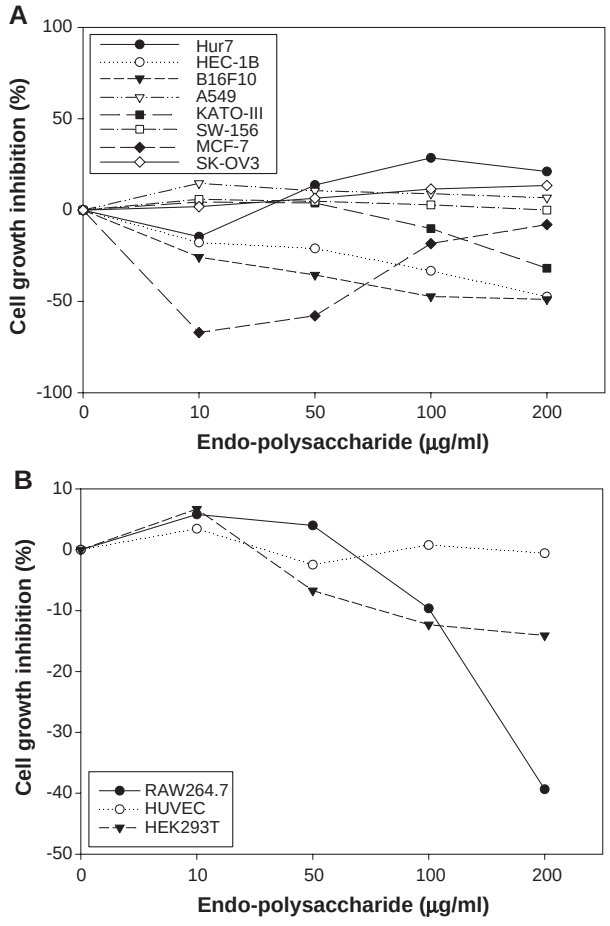

Fig. 9. The cytotoxic effect of purified endo-polysaccharide on various cancer cells (A) and normal cells (B). The polysaccharide was added to the cancer cells (5×10^4 cells/well) at final concentrations of 10, 50, 100, and 200 μg/ml. Cell growth inhibition was measured by the MTT colorimetric method at 48 h of cultivation. Data points represent the mean of triplicate repeats.

with the exception of Hur7 and MCF-7 (Fig. 9A). On the other hand, the endo-polysaccharide was not toxic against normal cells while a strong proliferation effect was observed for Raw264.7 murine macrophages (Fig. 9B). These results indicate that the endo-polysaccharide does not inhibit the growth of cancer cells directly but acts as a potent macrophage-stimulating agent for inhibition of tumor growth in the mouse peritoneal cavity.

Discussion

Kim et al. (2005) reported that the in vitro immuno-stimulating activity of water-soluble endo-polysaccharide isolated from *I. obliquus* mycelia is associated with functional stimulation of B-lymphocytes and macrophages. The nitrite production of purified endo-polysaccharide markedly increased compared to crude endo-polysaccharide, indicating that the purified endo-polysaccharides enhance phagocytosis and nitric oxide production in macrophages, resulting in immuno-stimulating activity. Thus, the anti-cancer effect of endo-polysaccharide in tumor-bearing mice is probably related to immuno-stimulation. Our results are in agreement with Kim et al. (2005) showing that endo-polysaccharide significantly activates the macrophage function of mouse immunocytes. The B16F10 murine melanoma cells which we used were a highly metastatic-malignant neoplasm of melanocytes. The endo-polysaccharide suppressed the in vivo growth of melanoma cells in mice after both oral and intraperitoneal administration with intraperitoneal being more effective. Mice that survived for 60 days did not show any tumor growth. Lee (1994) reported that oral administration of lentinan from *Lentinus edodes* or schizophyllan from *Schizophyllium commune* did not suppress tumor growth while peptidomannan (KS-2) from *L. edodes* mycelia effectively suppressed growth. In most cases, intraperitoneal administration is more rapid and effective than oral (Bae et al., 2005). However, it is unknown how orally administered substances are absorbed into the intestine. The anti-tumor action of endo-polysaccharide via oral administration is likely to involve multiple delivery processes (Carini et al., 2004).

An MTT assay has shown indirect cell-mediated cytotoxicity based on hydrolysis of MTT by mitochondrial dehydrogenases of living cells. We therefore tested direct cytotoxicity and/or a proliferation effect of endo-polysaccharide in both tumor and normal cells. Most cancer cells were unaffected by endo-polysaccharide, except for the Hur7 and MCF-7 cell lines. The endo-polysaccharide did not show any direct cytotoxic effect on melanoma cells at a dose of up to 200 μg/ml, and no cytotoxicity for normal cells. Similarly, Mizuno et al. (1999) reported that the polysaccharides extracted and fractionated from cultured mycelia of *I. obliquus* had weak inhibitory effects against cdc25 phosphatase, which participates in regulation of cancer cell cycle.

On the other hand, we found a proliferation effect for RAW 264.7 macrophages at a high dose. The proliferation process might activate in vivo tumor growth. Despite a marginal proliferation effect in vitro, we observed a strong endo-polysaccharide anti-tumor effect against B16F10 murine melanoma cells in vivo. This discrepancy suggests the existence of another action mode.

Ooi and Liu (2000) reported that polysaccharides from mushrooms exert anti-tumor effects via activation of different immune responses in the host rather than by directly attacking cancer cells. The effects may be obtained through an indirect pathway, such as activation of immunocytes in the mouse peritoneal cavity. Nonspecific host macrophage and natural killer cell defenses lead to suppression of tumor cell populations (Killion and Fidler, 1998). Since macrophages are obtained primarily from peritoneal exudates of mice (Kim et al., 2005), the in vivo anti-tumor effect of endo-polysaccharide is probably related to activation of macrophages rather than to direct cytotoxicity against tumor cells. It is believed that endo-polysaccharide can activate peritoneal macrophages and induce the production of host defense molecules, such as nitric oxide, and cytokines. Consequently, initial development of B16F10 melanoma cells is inhibited.

Erickson et al. (1980) reported that macrophages from melanoma-bearing mice were cytostatic and cytotoxic at an early stage of tumor growth. However, as the melanoma cells grew, the cytostatic effect of macrophages was gradually reduced. There was a loss or reduction in cytotoxicity. Thus, macrophages cannot completely suppress melanoma proliferation and they are relatively ineffective in controlling a large number of tumor cells. Previously, we reported (Kim et al., 2005) that endo-polysaccharide can induce the macrophage-enhanced mRNA expression of some inflammatory cytokines, including IL-1β, IL-6, iNOS and TNF-α. Administration of endo-polysaccharide enhanced the production of NO and tumoricidal cytokines by murine peritoneal macrophages at the initial phase of tumor development. The activated macrophages are believed to be involved in tumor cytotoxicity (Maeda et al., 1984). Kim et al. (2004) reported that an acidic polysaccharide from *Phellinus linteus*, belonging to family Hymenochataceae closely related to *I. obliquus*, exhibited in vitro peritoneal macrophage tumoricidal activity against B16F10 melanoma cells via stimulation of NO and TNF-α. Nitrite has been identified as the major effective molecule involved in destruction of tumor cells by activated macrophages (Lorsbach et al., 1993).

Bae et al. (2005) reported that a crude water-extract of *Phellinus gilvus* inhibited tumor growth and induced cell apoptosis in B16F10 melanoma-allografted mice. They observed in vivo inhibition of tumor growth at a dose of 100 mg/kg (i.p.), which is similar to the effect of adriamycin at a dose of 0.1 mg/kg. On the other hand, according to Han et al. (1999), endo-polysaccharide from mycelial cultures of *P. linteus* inhibited tumor growth at a dose of 100 mg/kg (i.p.), probably via immuno-stimulation in a B16F10 melanoma model. Han et al. (1998) reported that polysaccharide isolated from *Angelica gigas* caused a remarkable increase in the survival rate compared to a control group at a dose of 30 mg/kg (i.p.) in a B16F10 melanoma model. These observations

indicate that immunotherapy through activation of a host immune system can be a good alternative method for cancer control.

Polysaccharides with an anti-tumoral activity differ greatly in their chemical compositions, configurations, and physical properties. Although it is difficult to correlate polysaccharide structure with anti-tumor activity, some relationships can be inferred. Our purified endo-polysaccharide fraction, designated as FII-1, was a fucoglucomannan composed primarily of mannose with small amounts of glucose, fucose, and glucosamine. Mizuno et al. (1999) reported that water-soluble polysaccharide from the sclerotia of *I. obliquus* was a hetero-polysaccharide of xylogalactoglucan composed of glucose, mannose, galactose, xylose, arabinose, and fucose. Until now, there has been no information regarding endo-polysaccharide from cultivated mycelia of *I. obliquus*. Wasser (2002) reported that mannan was the major component of some active hetero-polysaccharides, e.g., α-(1→3)-mannans from *Dictyophora indusiata*, glucuronoxylomannan from *Tremella fuciformis*, glucomannan from *Agaricus blazei*, and galacto-glucomannan from *L. edodes*. Song et al. (1995) reported that hetero-polysaccharide isolated from the mycelia of *P. linteus* consisted primarily of mannose. The presence of protein has been reported to be important for biological activities (Mizuno et al., 1992) and protein is especially important for the biological activities of the hetero-polysaccharides. However, our results show that proteins combined with endo-polysaccharide do not seem to affect the activity (Kim et al., 2005). The polysaccharide linkage type is another important factor for anti-tumor or immuno-stimulating activity. Our endo-polysaccharide consists of α-linkages, according to ^{1}H NMR analysis. This structure is different from other known immuno-stimulating β-glucans (Usui et al., 1981; Mizuno et al., 1992). Several α-(1→3)-mannans from mycelia or fruit bodies of Jelly mushrooms such as *T. fuciformis* and *Tremella mesenterica* have been reported to have immuno-stimulating anti-tumor effect (Gao et al., 1996; Vinogradov et al., 2004). Especially, the activity was considered to depend chiefly on the backbone structure of α-(1→3)-D-mannans. A detailed structural analysis is needed.

Conclusion

α-Linked fucoglucomannan isolated from cultivated mycelia of *I. obliquus* can inhibit tumor growth in vivo. The endo-polysaccharide-mediated inhibition of tumor growth is apparently caused by an induced humoral immunity of the host defense system rather than by a direct cytotoxic effect against tumor cells. The endo-polysaccharide from *I. obliquus* mycelia has a potential for clinical use in cancer prevention and treatment.

Acknowledgement

This work was partially supported by funds from the Bio-consortium Center of KRIBB and the Sanhak Foundation in Korea.

References

Bae, J., Jang, K., Yim, H., Jin, H., 2005. Polysaccharides isolated from *Phellinus gilvus* inhibit melanoma growth in mice. Cancer Letter 218, 43–52.

Campbell, W.A., Davidson, R.W., 1938. A *Poria* as the fruiting stage of the fungus causing the sterile conks on birch. Mycologia 30, 553–560.

Carini, M., Aldini, G., Orioli, M., Piccoli, A., Rossoni, G., Facino, R.M., 2004. Nitric oxide release and distribution following oral and intraperitoneal administration of nitroaspirin (NCX 4016) in the rat. Life Sciences 74, 3291–3305.

Cui, Y., Kim, D.S, Park, K.C., 2005. Antioxidant effect of *Inonotus obliquus*. Journal of Ethnopharmacology 96, 79–85.

Erickson, K.L., McNeill, C.J., Gershwin, M.E., 1980. Tumoricidal activity of syngeneic macrophages from melanoma-bearing mice: changes with progressive tumor growth. Cancer Immunology, Immunotheraphy 9, 93–99.

Gao, Q.P., Jiang, R.Z., Chen, H.Q., Jensen, E., Seljelid, R., 1996. Characterization and cytokine stimulating activities of heteroglucans from *Tremella fusiformis*. Planta Medica 62, 297–302.

Han, S.B., Kim, Y.H., Lee, C.W., Park, S.M., Lee, H.Y., Ahn, K.S., Kim, I.H., Kim, H.M., 1998. Characteristic immunostimulation by angelan isolated from *Angelica gigas* Nakai. Immunopharmacology 40 (1), 39–48.

Han, S.B., Lee, C.W., Jeon, Y.J., Hong, N.D., Yoo, I.D., Yang, K.H., Kim, H.M., 1999. The inhibitory effect of polysaccharides isolated from *Phellinus linteus* on tumor growth and metastasis. Immunopharmacology 41, 157–164.

Ichimura, T., Otake, T., Mori, H., Maruyama, S., 1999. HIV-1 protease inhibition and anti-HIV effect of natural and synthetic water-soluble lignin-like substance. Bioscience, Biotechnology, and Biochemistry 63 (12), 2202–2204.

Jarosz, A., Skorska, M., Rzymowska, J., Kochmanska-Rdest, J., Malarczyk, E., 1990. Effect of the extracts from fungus *Inonotus obliquus* on catalase level on Hela and nocardia cells. Acta Biochimica Polonica 37 (1), 149–151.

Kahlos, K., 1994. Antifungal activity of cysteine, its effect on C-21 oxygenated lanosterol derivatives and other lipid in *Inonotus obliquus*, in vitro. Applied Microbiology and Biotechnology 3, 339–385.

Kahlos, K., Kangas, L., Hiltunen, R., 1987. Antitumor activity of some compounds and fractions from an *n*-hexane extract of *Inonotus obliquus*. Acta Pharmaceutica Fennica 96, 33–40.

Killion, J.J., Fidler, I.J., 1998. Therapy of cancer metastasis by tumoricidal activation of tissue macrophages using liposome-encapsulated immuno-modulator. Pharmacology and Therapeutics 78, 141–154.

Kim, G.Y., Choi, G.S., Lee, S.H., Park, Y.M., 2004. Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. Journal of Ethnopharmacology 95, 69–76.

Kim, Y.O., Han, S.B., Lee, H.W., Ahn, H.J., Yoon, Y.D., Jung, J.K., Kim, H.M., Shin, C.S., 2005. Immuno-stimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*. Life Sciences 77 (19), 2438–2456.

Lee, J.H., 1994. Anti-tumor and immunostimulating activity of fungal polysaccharide. Microorganisms and Industry 20, 14–21.

Lorsbach, R.B., Murphy, W.J., Lowenstein, C.J., Synder, SH., Russell, S.W., 1993. 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. Journal of Biological Chemistry 268, 1908–1913.

Maeda, Y.Y., Suga, T., Watanabe, S.T., Rokutanda, M., Chihara, G., 1984. Induction of early acute phase serum factors associated with inflammation by Lentinan. In: Aoki, T., Subra, E., Urushizaki, I. (Eds.), Manipulation of Host Defence Mechanisms. APCS, vol. 38. Excerpta Medica.

Mizuno, T., 1999. The extraction and development of antitumor-active polysaccharides from medicinal mushrooms in Japan. International Journal of Medical Mushrooms 1, 9–29.

Mizuno, T., Ando, M., Sugie, R., Ito, H., Shimura, K., Sumiya, T., Matsuura, A., 1992. Antitumor activity of some polysaccharides isolated from an edible mushroom, *Ningyotake*, the fruiting body and the cultured mycelium of *Polyporous confluens*. Bioscience, Biotechnology, and Biochemistry 56 (1), 34–41.

Mizuno, T., Zhuang, C., Abe, K., Okamoto, H., Kiho, T., Ukai, S., Leclerc, S., Meijer, L., 1999. Antitumor and hypoglycemic activities of polysaccharides from the sclerotia and mycelia of *Inonotus obliquus* (Pers.:Fr.) Pil. (Aphyllophoromycetideae). International Journal of Medical Mushrooms 1, 301–316.

Ooi, V.E., Liu, F., 2000. Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. Current Medical Chemistry 7, 715–729.

Pagè, M., Bejaoui, N., Cinq-Mars, B., Lemieux, P., 1988. Optimization of the tetrazolium-based colorimetric assay for the measurement of cell number and cytotoxicity. International Journal of Immunopharmacology 10, 785–793.

Saar, M., 1991. Fungi in Khanty fork medicine. Journal of Ethnopharmacology 31 (2), 175–179.

Solomon, P.W., Alexander, L.W., 1999. Therapeutic effects of substances occurring in higher *Basidiomycetes* mushrooms: a modern perspective. Immunology 19, 65–96.

Song, K.S., Cho, S.M., Lee, J.H., Kim, H.M., Han, S.B., Ko, K.S., Yoo, I.D., 1995. B-lymphocyte-stimulating polysaccharide from mushroom *Phellinus linteus*. Chemical and Pharmaceutical Bulletin 43 (12), 2105–2108.

Usui, T., Iwasaki, Y., Hayashi, K., Mizuno, T., Tanaka, M., Shinkai, K., Arakawa, M., 1981. Antitumor activity of water-soluble β-D-glucan elaborated by *Ganoderma applanatum*. Agricultural and Biological Chemistry 45 (1), 323–326.

Vinogradov, E., Petersen, B.O., Duus, J.Ø., Wassor, S.P., 2004. The isolation, structure, and applications of the exocellular heteropolysaccharide glucuronoxylomannan produced by yellow brain mushroom *Tremella mesenterica* Ritz.: Fr. (Heterobasidiomycetes). International Journal of Medicinal Mushrooms 6, 335–345.

Wasser, S.P., 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology 60, 258–274.