Curr Microbiol (2008) 57:454–462
DOI 10.1007/s00284-008-9233-6

Identification of *Inonotus obliquus* and Analysis of Antioxidation and Antitumor Activities of Polysaccharides

Yana Song · Jing Hui · Wei Kou · Ru Xin · Fei Jia · Ning Wang · Fengqing Hu · Huili Zhang · Hongsheng Liu

Received: 7 April 2008 / Accepted: 28 July 2008 / Published online: 16 September 2008

Abstract *Inonotus obliquus*, a wild wood-decay fungus which grows on Betula trees in cool climates, has a variety of biological activities that the scientific community is paying more and more attention to. However, the research work is moving at a snail's pace. The methods of strain identification and the hypha microstructure have not been reported. We isolated one strain of filamentous molds from fruit body which was collected from birch wood on Changbai Mountain, cultivated mycelia on an inclined plane, and examined its micromorphology based on macroscopic examination. The strain was identified as *I. obliquus* by sequencing its ITS (internal transcribed spacer) domain. We subsequently investigated some of the mycelium polysaccharides' biological activities. The strain used in this study as the producers of antioxidation and anticancer polysaccharides was LNUF008. After fermentation in a 30-L fermenter, mycelia were obtained. The polysaccharides were extracted by transonic recirculation and ethanol precipitation. In order to identify the antioxidation effect, we designed an assay to test the inhibition of endogenous and Fe^{2+}-Cys-induced lipid peroxidation as well as ferrous sulfate/ascorbate (Fe^{2+}-VC)-induced mitochondrial swelling. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method was used to study the antiproliferation activity of the polysaccharides on SMMC7721 hepatoma cells. The results indicate that *I. obliquus* polysaccharides exhibit high antitumor and antioxidation effects. The submerged culture method of growing *I. obliquus* will enable large-scale production of the polysaccharides.

Y. Song · J. Hui · W. Kou · R. Xin · F. Jia · N. Wang · F. Hu · H. Zhang · H. Liu (✉)
School of Life Science, Liaoning University, Chongshan Middle Road 66, Shenyang 110036, People's Republic of China
e-mail: liuhongsheng@lnu.edu.cn

Introduction

Inonotus obliquus (chaga) is a white rot fungus that belongs to the phylum Basidiomycota. It is a typical tree disease fungus widely distributed over Europe, Asia, and North America that grows on birch trees in colder northern climates [18, 26]. Chaga is a fungal parasite, sucking nutrients out of living trees rather than from the ground. It is often found as a sterile conk (sclerotium). Since the sixteenth century, chaga has been used as a folk remedy in Russia and western Siberia [7, 9].

In recent years, more than 20 different kinds of bioactive components have been found in chaga, such as triterpenoids [25] inotodiol, trametenolic acids, and lanosterol [30]. Many biological activities have also been attributed to chaga, including high antitumor [9, 20] anti-HIV [3], antioxidation [5, 17, 23] antimitotic [2] hypoglycemic [29] and anti-inflammatory activities [24] and it doesn't show any side effects in treatment of cancers and digestive system diseases.

DNA sequence data of 18S rRNA, 26S rRNA, ITS, and mitochondrial rRNA have frequently been used in recent phylogenetic studies of eukaryotic cells. Among them, the ITS domain, which has the highest evolutionary speed, is appropriate for the molecular identification or delimitation of fungi and is now commonly used in the systematics of species within a genus [13]. We used the phylogenetic analysis of ITS sequences compared to registered sequences of Basidiomycota in the GenBank, EMBL, DDBJ, and PDB databases to identify the LNUF008 strain.

Fruit body, basidiospore, and mycelium morphology are important characteristics for identifying various kinds of fungi [6]. Plenty of papers have described the thalline appearance of *I. obliquus*, but little work has been done to study the microstructure of the mycelia. In this study, the morphological characteristics of laboratory-cultivated *I. obliquus* mycelia were examined using fluorescence microscopy in order to fully understand the microstructure of *I. obliquus* and provide new identification criteria.

Many polysaccharides, such as *lentinan*, *schizophyllan*, *krestin*, and those found in *Ganoderma* [27], have been used in clinical cancer therapies as well as other applications [1, 16]. In recent years, an increasing number of pharmaceutical companies have begun producing functional mushroom polysaccharides from fermental mycelia [33] instead of fruit bodies because of the high productivity and low cost [10, 19]. Our polysaccharide producer is the mycelium, which is isolated from the fruit bodies collected at Changbai Mountain in China. We extract the *I. obliquus* polysaccharides from the submerged fermental mycelia. At present, because of the lack of studies on the fermentation of *I. obliquus* mycelia, we only have very limited knowledge about the polysaccharide production level from this source.

After obtaining the *I. obliquus* polysaccharides from the submerged fermental mycelia, we identified its antioxidation and antitumor activities [32, 34]. For the antioxidation effect, we tested the inhibition to endogenous and Fe^{2+}-Cys-induced lipid peroxidation and ferrous sulfate/ascorbate (Fe^{2+}-VC)-induced mitochondrial swelling. No literature has documented the antioxidation activities of *I. obliquus* polysaccharides using this method. In this project, the antitumor effect was confirmed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. This activity is likely due to the antiproliferation of the polysaccharides in SMMC7721 hepatoma cells.

In the end, we compared the antioxidation and antitumor activities of fermental mycelia with that of fruit body of *I. obliquus*, by substituting the fermental mycelial polysaccharides with those from the fruit body. This experiment provided some valuble data for future polysaccharide production.

Materials and Methods

Chemicals and Biological Compounds

I. obliquus fruit bodies used in our experiment were harvested from the northern forest in China. The strains were isolated from the fruit bodies, maintained on potato dextrose agar (PDA) slants at 30°C for fifteen days, and stored at 4-C All chemicals and solvents were of analytical grade. Kunming mice were purchased from Shenyang Medical College (each weighing 10–18 g).

Microexamination

The mycelia in the PDA slant were inoculated onto a 60-mm-diameter PDA plate, which was incubated 20 days at 30°C. Small pieces of the colony were removed, pressed onto a glass microscope slide, observed under a Nikon light microscope, and photographed. The micromorphology of aerial hyphae and intrahyphae was obtained and measurements were taken.

ITS Domain Identification

We picked the cultured mycelia from the PDA slant to make the lysate and degenerated it at 85°C. Then the TaKaRa Fungi Identification PCR Kit was used for PCR amplification with 1 μl of the lysate as the template, and 5 μl of the PCR products was separated by agarose gel electrophoresis. After that, we cut the gel in order to recover the objective fragments using the TaKaRa Agarose Gel DNA Purification Kit Ver.2.0, and separated by agarose gel electrophoresis once more. The rDNA ITS sequence of isolate of *I.obliquus* was sequenced with the corresponding primers (TaKaRa Biotechnology Co., Ltd.), and the sequences obtained were compared with those stored in the GenBank data system with BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) including the GenBank, EMBL, DDBJ, and PDB databases [2]. Another molecular identification method was accomplished using phylogenetic tree analysis of 18 Basidiomycota ITS nucleotide sequences in the GenBank database.

Preparation of Polysaccharides

In our study, an improved PDA liquid medium (used as both seed and fermental broth) that included 20 g of glucose, 5 g of proteose peptone, 1.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of K_2HPO_4, and 0.4 g of KH_2PO_4 was added to 1 l of water that had been used to boil 200 g of potatoes.

Seed cultivations were carried out in a 500-ml flask containing 100 ml of liquid medium on a shaking incubator at 30°C and 180 rpm for 7 days. These were followed by fermental cultivations which were done in a 30-L fermenter. The medium for the fermental cultivations consisted of 400 ml seed cultures and 19.6 l PDA liquid medium. After cultivation for 7 days, the *I. obliquus* mycelia were collected by broth filtration at 5000 rpm for 20 min and were washed with distilled water to clean the surface polysaccharides. They were then dried at 40°C for 1 h [28].

The desiccated mycelia were resuspended in 20 vol of distilled water and extracted at 121°C for 5 h in an autoclave sterilizer. After centrifugation at 4000 rpm for 10 min, the supernatant was concentrated, followed by the addition of 3 vol of ethanol to precipitate the polysaccharides. The *I. obliquus* polysaccharides were obtained by centrifugation at 8000 rpm for 15 min and dried.

In order to compare the antioxidation and antitumor activities of fermental mycelial polysaccharides with those of fruit bodies. We extracted the polysaccharides from the fruit body powder using the same methods mentioned above.

Investigating the Inhibition of Lipid Peroxidation

In this study, two assays were performed to research the inhibitive activities of endogenous and Fe^{2+}-Cys-induced lipid peroxidation by *I. obliquus* polysaccharides. After mice were fasted for twelve hours, they were sacrificed by cervical dislocation. The liver was rapidly removed and its homogenates were prepared with 50 vol of normal saline at 4°C Four reaction systems containing 2 ml of liver homogenate and 1 ml of polysaccharide solution at different concentrations (100, 200, 300, and 400 μg/ml) as well as a control with only normal saline were set up. All of them were kept in a 37°C water bath for 1.5 h, and 2 ml of 10% (w/v) trichloroacetic acid (TCA) was added to terminate each reaction. When 2 ml of 0.6% (w/v) thiobarbituric acid (TBA) was added, the reaction systems were boiled for 15 min to develop the coloration. After cooling and centrifugation, OD values were measured at 532 nm. We calculated the inhibitive rate of endogenous lipid peroxidation as Inhibitive Rate (%) = $(OD_c - OD_s)/OD_c \times 100\%$, where OD_c and OD_s are the OD values of the control and the sample, respectively.

The inhibitive effect of Fe^{2+}-Cys-induced lipid peroxidation by *I. obliquus* polysaccharides was demonstrated using a similar assay. We prepared four reaction systems, then a cocktail reagent of 50 μl of 0.01 M Cys and 100 μl of 0.001 M $FeSO_4$ was added to reactions. Subsequent procedures, including the development of coloration, measurements of OD values, and calculation of the inhibition rate, were the same as described above.

Detecting the Depression Effect of Mitochondrial Swelling

After removal of blood, mice livers were washed promptly with cold normal saline, weighed, and homogenized in 10 vol of 0.25 M sucrose in an ice bath. The homogenates were clarified by centrifugation at 1000 rpm for 20 min at 4°C. The mitochondria were subsequently collected by further centrifugation of the former supernatants at 10,000 rpm for 20 min, washed twice with 20 mM Tris-HCl buffer (pH 7.4), resuspended in the same buffer to a concentration of 0.5 mg/ml of protein, and stored at 0°C. Each of the reaction systems included 2.5 ml of mitochondrial suspension and 1 ml of polysaccharide solution (at 100, 200, 400, and 800 μg/ml). One milliliter of normal saline was used as two controls (a positive control with only normal saline and a negative with all the reagents). We added 50 μl of 1 mM $FeSO_4$ and 50 μl of 1 mM VC (vitamin C) solution to the polysaccharide reaction systems and the negative control and incubated at room temperature for 20 min.

The damage caused by mitochondrial swelling was determined by measuring the OD values at 520 nm. The results of the inhibitive rates of Fe^{2+}-VC-induced mitochondrial swelling are expressed as Inhibition Rate = $(OD_s - OD_n)/(OD_p - OD_n) \times 100\%$, where OD_s, OD_p, and OD_n are the OD values of the sample, the positive control, and the negative control, respectively.

Assay of Antitumor Activity

The hepatoma cell line, SMMC7721, was used to analyze the direct cytotoxicity of *I. obliquus* polysaccharides, which was incubated in a 5% CO_2 incubator at 37°C and kept in RPMI-1640 with 10% calf serum. To investigate the inhibitory effect of endopolysaccharides against the hepatoma tumor, a cytotoxicity test was performed. We adjusted the cancer cell concentration to 3×10^5 cells/ml, added 100 μl of the suspension to each well of a 96-well plate, and aliquoted the medium to four groups. Group 1 (G1), with no cell suspension, contained only 100 μl of normal saline + 10 μl of normal saline. Group 2 (G2) included 100 μl of cell suspension and 10 μl of mitomycin (1, 2, 4, and 8 μg/ml). Group 3 (G3) consisted of 100 μl of cell suspension and 10 μl of endopolysaccharide solution. Group 4 (G4) contained only 100 μl of cell suspension and 10 μl of normal saline. After 44 h of incubation, the MTT assay was done, followed by OD measurements at 540 nm using the micro-plate reader. We subsequently calculated the medicine inhibition ratio and the cell viability ratio.

Antioxidation and Antitumor Activity Comparison

In this study, we used the method of water decoction and ethanol precipitation to extract polysaccharides from the *I. obliquus* fruit body. To compare the antioxidation and antitumor activities of the two polysaccharides which were extracted from the mycelia versus the fruit body, the same experiments were repeated with fruit body polysaccharides. The entire procedures were invariable.

Results

Microstructure of Mycelia

Colony diameters on the PDA plate were 12 to 16 mm at 7 days of incubation and 30 to 35 mm at 14 days. They looked moderately thick, raised, and densely woolly, and the color turned yellow. When incubated for 21 days at 30°C, the colony diameters were 77 to 82 mm, which was a little brown (Fig. 1a), and the agar beneath the mycelia was stained a dark brown or reddish brown. Then we observed the microstructure of the *I. obliquus* mycelia under a Nikon visible-light microscope. The micromorphology of aerial hyphae and intrahyphal hyphae is shown in the following photomicrographs. All of the hyphae lacked clamp connections. Aerial hyphae were hyaline and 3.0–5.0 μm wide but turned brown with growth (Fig. 1b). Intrahyphal hyphae were brown, 1.7–6.5 μm wide, and thick walled (Fig. 1c).

Molecule Identification of the Strain

The internal transcribed spacer was amplified (Fig. 2). Subsequent sequence determination and phylogenetic evaluation were also done following the methods mentioned above. In the phylogenetic analysis of ITS sequences, the new sequences of LNUF008 were a 100% (686 of 686) match to the sequences of *I. obliquus* DQ103883 and AY558593 strains in GeneBank database. This suggested that the causal fungus was actually a strain of *I. obliquus*.

Comparing with 18 Basidiomycota ITS nucleotide sequence in the GenBank database, a phylogenetic tree analysis of ITS sequences was carried out, which suggested that the isolate LNUF008 strain was statistically identical to *I. obliquus* (Fig. 3).

Optimal Conditions for Extraction of Polysaccharides

We first determined the submerged culture conditions by modifying the ingredients of fermental broth (Table 1). A modified PDA broth was used in this study with 20 g of glucose, 5 g of proteose peptone, 1.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of K_2HPO_4 and 0.4 g of KH_2PO_4, which were added to 1L of the water used to boil 200 g of potatoes. The

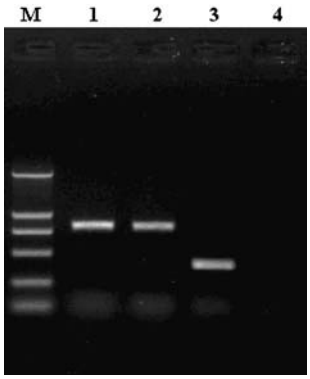

Fig. 2 Products of PCR amplification. M, DNA marker DL2000; lanes 1 and 2, LNUFOO8; lane 3, positive control; lane 4, negative control

I. obliquus polysaccharides were extracted in the temperature range of 105–135°C over a period of 3 to 8 h. As the temperature and time were increased, the amount of polysaccharides recovered also increased. The best extraction conditions we tested were 121°C for 5 h (Fig. 4).

Inhibitory Effect on Lipid Peroxidation of *Inonotus obliquus* Polysaccharides

Inhibitory activities of *I. obliquus* mycelial polysaccharides on lipid peroxidation were found to block endogenous and Fe^{2+}-Cys-induced lipid peroxidation. We found that the polysaccharides can restrain endogenous lipid peroxidation at a low concentration, and the inhibitive rate increased with increasing concentration of the polysaccharides in a dose-dependent manner, when below 300 μg/ml. The inhibitory rate of Fe^{2+}-Cys-induced lipid peroxidation was subsequently detected and also increased with increasing concentrations of the polysaccharides. The highest inhibitive rate was at 200 μg/ml. However, inhibitory effects on Fe^{2+}-Cys-induced lipid peroxidation were exhibited at a much lower concentration than on endogenous lipid peroxidation (Fig. 5).

Inhibitory Activity to Mitochondrial Swelling

As expected, lower OD_{520} values were recorded in negative controls because the Fe^{2+}-VC system induces oxidative

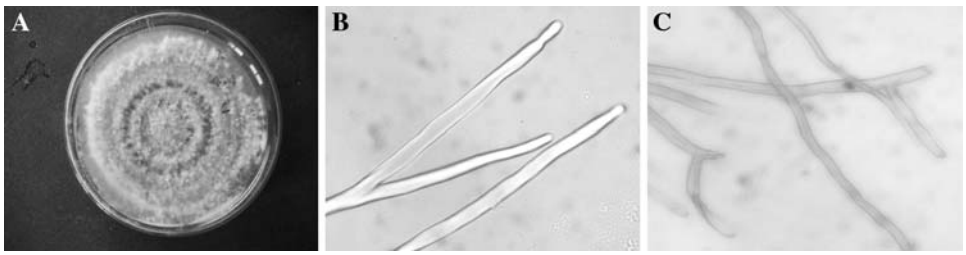

Fig. 1 The mycelia of *Inonotus obliquus*. (**a**) Colony of the case isolated on a 90-mm-diameter PDA plate after 21 days of incubation at 30°C. (**b**) Aerial hyphae of strain LNUF008. (**c**) Intrahyphae of strain LNUF008

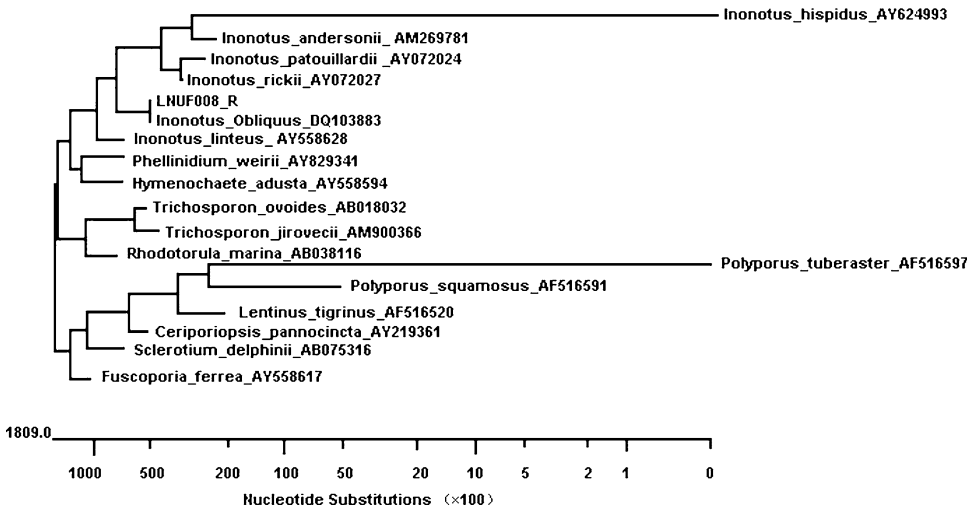

Fig. 3 Phylogenetic tree analysis of 18 Basidiomycota ITS nucleotide sequences

Table 1 Mycelial growth in shaking flasks containing fermentation broth augmented with various compounds

	Ingredient of fermentation broth									Mycelial dry weight (g/l)
	Glucose (2%)	Proteose peptone (0.5%)	Malt extract (0.6%)	Yeast extract (0.3%)	$MgSO_4 \cdot 7H_2O$ (0.15%)	K_2HPO_4 (0.02%)	KH_2PO_4 (0.04%)	Boiled potato water	Distilled water	
A	+	−	−	−	−	−	−	+	−	10.04
B	+	+	−	−	−	−	−	−	+	2.97
C	+	+	−	−	−	−	−	+	−	14.47
D	+	+	−	−	+	+	+	+	−	15.22
E	−	−	+	+	−	−	−	+	−	11.35
F	−	−	+	+	+	+	+	−	+	5.78
G	−	+	+	−	−	−	−	+	−	9.10
H	−	+	+	−	+	+	+	+	−	8.36
I	−	+	+	+	−	−	−	−	+	4.49
J	−	+	+	+	+	+	+	−	+	3.92
K	−	+	+	+	−	−	−	+	−	10.28

Note: Data were obtained by averaging three replications

mitochondrial membrane swelling. *I. obliquus* polysaccharides inhibited the above-mentioned mitochondrial damage. Measuring at higher OD_{520} values compared to the negative control, we found that the polysaccharide concentrations inhibited mitochondrial swelling in a dose-dependent manner (Table 2).

Cytotoxicity of Polysaccharides Against Cancer Cells

The antitumor activity of *I. obliquus* mycelia polysaccharides was investigated by an MTT assay. Mitomycin is an anticancer medicine which strongly inhibits the hepatoma cell line, SMMC7721. We did a comparison study between the anticancer activity of mitomycin and that of *I. obliquus* polysaccharides. Figure 6 shows the survival rate of cells and the inhibitive rate of the medicine. These results indicate that *I. obliquus* polysaccharides can restrain the growth of cancer cells. The *I. obliquus* polysaccharide antitumor activity was measured and it showed the highest inhibitive rate at the dose of 150 μg/ml, that is, the same as the inhibitive rate of mitomycin at a dose of 5 μg/ml. In view of the strongly effective antitumor ability of mitomycin, the anticancer effect of *I. obliquus* polysaccharide is verified.

Comparison of Activities of Polysaccharides from Different Sources

We used the same methods to detect the antioxidation and antitumor activities of the polysaccharides of the fruit body, keeping the polysaccharides' quantity invariable. Then we compared the antioxidation and antitumor activities of the

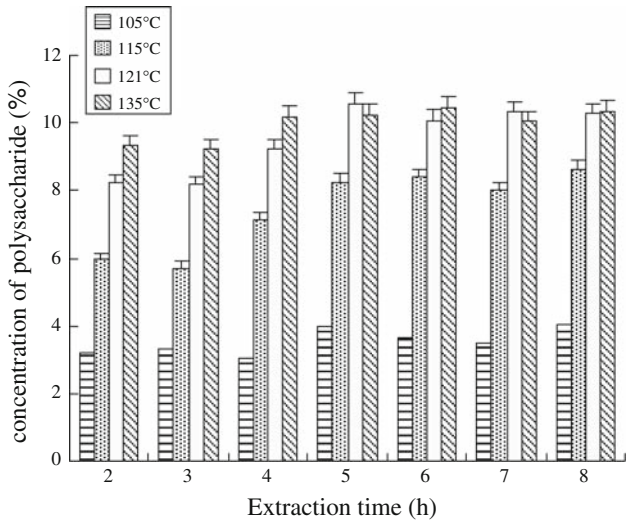

Fig. 4 Effect of optimal conditions for extraction of polysaccharide

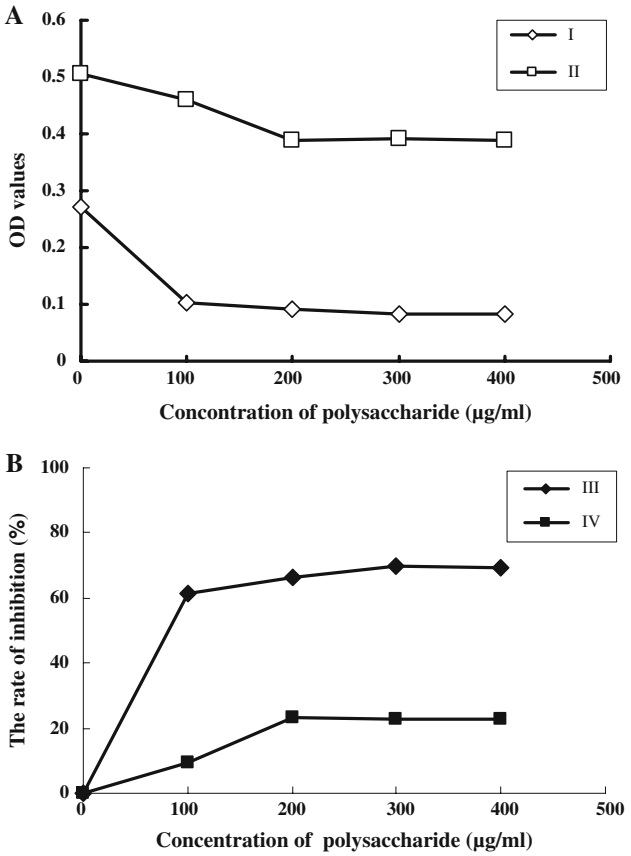

Fig. 5 Activity of polysaccharide inhibitory to endogenous and Fe^{2+}-Cys-induced lipid peroxidation. (**a**) OD value. (**b**) Inhibitory rate. I and III, endogenous lipid peroxidation; II and IV, Fe^{2+}-Cys-induced lipid peroxidation

polysaccharides from the two sources, respectively. The polysaccharides extracted from the mycelia showed stronger inhibitive activities to endogenous lipid peroxidation than

Table 2 Effect of inhibitory activity on mitochondrial swelling

Sample concentration	OD_{520}	Inhibitory rate (%)
Negative control (with Fe^{2+}-VC)	0.470 ± 0.021	–
100 μg/ml	0.500 ± 0.014	27.27
200 μg/ml	0.518 ± 0.018	34.55
400 μg/ml	0.520 ± 0.018	45.45
800 μg/ml	0.523 ± 0.010	48.18
Positive control (no Fe^{2+}-VC)	0.580 ± 0.014	–

Note: Data represent the average of three replications

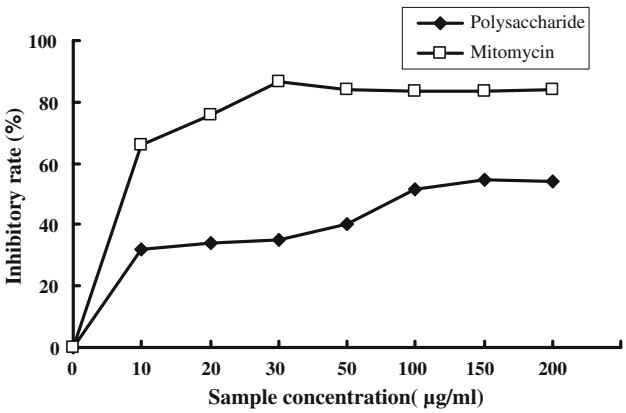

Fig. 6 Contrast of the activity inhibitory to hepatoma SMMC7721 cell survival

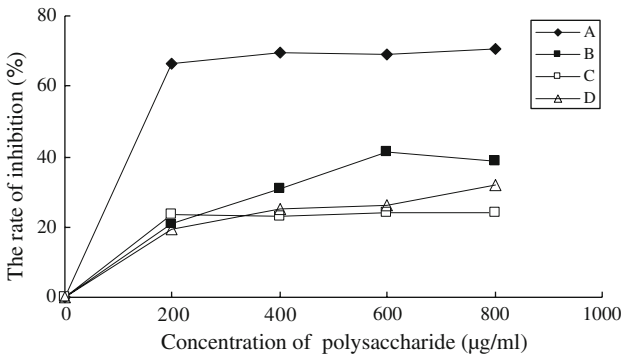

Fig. 7 Comparison of inhibitory activities of polysaccharides extracted from two sources to endogenous and Fe^{2+}-Cys-induced lipid peroxidation. (**a**) Inhibitory activities to endogenous lipid peroxidation of mycelial polysaccharide. (**b**) Inhibitory activities to endogenous lipid peroxidation of fruit body polysaccharide. (**c**) Inhibitory activities to Fe^{2+}-Cys-induced lipid peroxidation of mycelial polysaccharide. (**d**) Inhibitory activities to Fe^{2+}-Cys-induced lipid peroxidation of fruit body polysaccharide

those from the fruit body. Mycelial polysaccharides at 300 μg/ml reached the highest inhibitive rate of 69.74%, however, 600 μg/ml polysaccharides from the fruit body reached a highest inhibitive rate of only 41.27%. Figure 7 showed that the two sources of *I. obliquus* polysaccharides had similar inhibitive effects on Fe^{2+}-Cys-induced lipid

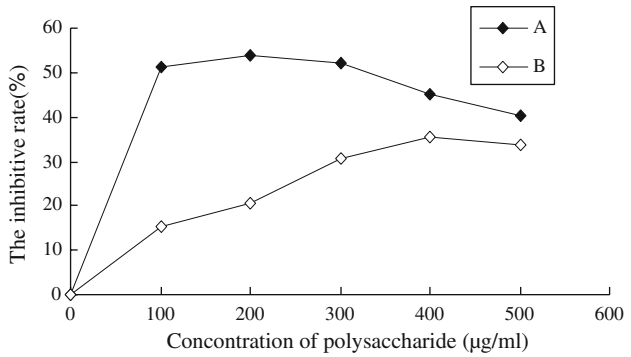

Fig. 8 The inhibitory rate of *I. obliquus* polysaccharides extracted from two sources to hepatoma SMMC7721 cells. (**a**) Mycelial polysaccharide. (**b**) Fruit body polysaccharide

peroxidation, and the polysaccharides from the fruit body were a little stronger. After the direct cytotoxicity experiment of *I. obliquus* polysaccharides which were extracted from mycelia and fruit body, we found that the mycelial polysaccharides had a more powerful ability to inhibit the tumor cell (Fig. 8), which reached the highest inhibitive rate at the dose of 150 μg/ml, compared to the dose of 400 μg/ml for fruit body polysaccharides.

Discussion

Inonotus obliquus is a medicinal fungus used as a folk remedy for almost five centuries, and is now attracting the attention of the scientific community. Previously, many investigations have been carried out to study its effect on cancer, digestive diseases, oxidation, and other conditions. Burczyk et al. [2] showed the cytotoxic effect of two aqueous extracts of *I. obliquus* on human cervical uterine cancer cells (Hela S3) in vitro. Rzymowska [15] reported the effect of aqueous extracts from *I. obliquus* on the mitotic index and enzyme activities. It was concluded that the *I. obliquus* extract inhibited the growth of tumor cells. The fungal extract caused a decrease in total cell protein and the mitotic index value. Babitskaia [5] found that *I. obliquus* synthesized phenolic pigments in the melanin family that had strong antioxidant and genoprotective effects. Cui et al. [23] discovered that four extracts from *I. obliquus* demonstrated antioxidant activity, superoxide, and peroxyl radicals. Together, these reports provided inspiration for our team to study *I. obliquus* further.

However, the references above were only related to the bioactivity of the sclerotia extraction. We found that the growth of the mushroom fruit bodies is very time-consuming. If we produce extractions exclusively from the sclerotia, the amount of polysaccharides that can be produced is relatively small. Therefore, we chose to cultivate mycelia, with the prospect of obtaining larger amounts of the polysaccharides.

I. obliquus belongs to the family Hymenochaetaceae of Basidiomycetes. Until now, few reports have shown any method for its identification. For this reason, we designed experiments in two directions in order to identify the new strain. On one hand, we observed the microstructure of mycelia; on the other hand, we conducted molecular identification. After culturing the mycelia, preparing the pressed slide, and taking photomicrographs of the aerial hyphae and intrahyphal hyphae, we gained better insight into these microstructures. The colony turned brown as the culture grew older. All of the hyphae lacked clamp connections. Aerial hyphae were hyaline, but intrahyphal hyphae were thick walled and brown.

ITS domain identification was carried out. After PCR amplification and gene sequencing, we obtained the ITS sequence of the LNUF008 strain. First, we compared it with those stored in the GenBank database using BLAST alignment software. The new sequences had 100% similarity in sequences of *I. obliquus* strains in GeneBank. Second, we used phylogenetic tree analysis of 18 Basidiomycota ITS nucleotide sequences in GenBank to identify the phylogenetic relationship. All results proved that the LNUF008 was actually a strain of *I. obliquus*.

Polysaccharides of edible mushrooms have a number of compounds with high bioactivities, such as lentinan, schizophyllan, and krestin [8, 11, 21, 22]. Furthermore, the medical effects are similar between the aqueous extract of fruit body and that of mycelia [14]. Li [10] reported that the fruiting body of *Cordyceps sinensis* in its caterpillar host shows a close resemblance in main constituents and antioxidation activity to the polysaccharides of *I. obliquus*. Using this information, in addition to our own methods and observations, we designed this project, including the test for measuring the anticarcinogenic activity against SMMC7721 hepatoma cells [12], determining the inhibitory effect to endogenous and Fe^{2+}-Cys-induced lipid peroxidation, and measuring the ability to iinhibit Fe^{2+}-VC-induced mitochondrial swelling [4, 31]. All of our experiments are reported here first. We identified the antitumor activity of *I. obliquus* polysaccharides by the MTT method, and determined the high level of inhibition of SMMC7721 cell survival (at a dose of 150 μg/ml). Assays of antioxidation showed that the polysaccharides were capable of inhibiting endogenous and Fe^{2+}-Cys-induced lipid peroxidation. The optimal amount of polysaccharides was 300 μg/ml to inhibit endogenous lipid peroxidation, and 200 μg/ml against Fe^{2+}-Cys-induced lipid peroxidation. The concentration that showed inhibitory effects on Fe^{2+}-Cys-induced lipid peroxidation was much lower than that for endogenous lipid peroxidation. Finally, we detected the antioxidation activity of

polysaccharides by measuring their ability to reduce mitochondrial membrane swelling and found that inhibitory activity to mitochondrial swelling was dose-dependent over the range of polysaccharide concentrations tested.

Because the resource of *I. obliquus* sclerotia is facing exhaustion, we hope that the polysaccharides extracted from the mycelia can serve as a good substitute for sclerotia polysaccharides. So antioxidation and anticancer assays were done with mycelial polysaccharides and fruit body polysaccharides, respectively. They showed that the mycelial polysaccharides had stronger activities of inhibition to endogenous lipid peroxidation and SMMC7721 hepatoma cells, and the two sources of polysaccharides had similar abilities against Fe^{2+}-Cys-induced lipid peroxidation.

I. obliquus has many medical functions. Polysaccharides extracted from the mycelia have a potential for clinical use, as they might be a good substitute for sclerotia polysaccharides, which are difficult to cultivate. Therefore, a detailed analysis is needed. This study provides information for further research and describes a novel method to produce larger amounts of the polysaccharides.

Acknowledgments This work was supported by the National Natural Science Fund (No. 30671852), Exploitation of Intellectual Resources Special Fund in Shenyang (No. 2006058), Science and Technology Government Department Fund in Liaoning province (No. 20062047), and Open Research Fund Program of the State Key Laboratory of Virology of China (No. 2007007).

References

1. Babitskaia VG, Shcherba VV, Ikonnikova NV (2000) Melanin complex of the fungus *Inonotus obliquus*. Prikl Biokhim Mikrobiol 36:439–444
2. Burczyk J, Gawron A, Slotwinska M, Smietana B, Terminska K (1996) Antimitotic activity of aqueous extracts of *Inonotus obliquus*. Boll Chim Farm 135:306–309
3. Cui Y, Kim DS, Park KC (2005) Antioxidant effect of *Inonotus obliquus*. J Ethnopharmacol 96:79–85
4. Ebina T (2002) Activation of antitumor immunity by intratumor injection of biological preparations. Gan To Kagaku Ryoh 35:115–134
5. Golovko VA (1999) Effects of bioglycans from birch fungus *Inonotus obliquus* on the electrical activity of cells from the cardiac venous sinus of the frog. Biull Eksp Biol Med 128:264–266
6. Hamel E (1996) Antimitotic natural products and their interactions with tubulin. Med Res Rev 16:207–231
7. Han SB, Lee CW, Jeon YJ, Hong ND, Yoo ID, Yang KH, Kim HM (1999) The inhibitory effect of polysaccharides isolated from *Phellinus linteus* on tumor growth and metastasis. Immunopharmacology 41:157–164
8. Hyun KW, Jeong SC, Lee DH, Park JS, Lee JS (2006) Isolation and characterization of a novel platelet aggregation inhibitory peptide from the medicinal mushroom, *Inonotus obliquus*. Peptides 27:1173–1178
9. Ichimura T, Watanabe O, Maruyama S (1998) Inhibition of HIV-1 protease by water-soluble lignin-like substance from an edible mushroom, *Fuscoporia oblique*. Biosci Biotechnol Biochem 62:575–577
10. Jalc D, Siroka P, Ceresnakova ZZ (1997) Effect of six species of white-rot basidiomycetes on the chemical composition and rumen degradability of wheat straw. J Gen Appl Microbiol 43:133–137
11. 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 Biochim Polonica 37:149–151
12. Kabbaj WB, Reheret S, Guimberteau J, Talou T, Olivier JM, Bensoussan M, Sobal M, Roussos S (2002) Comparison of volatile compound production in fruit body and in mycelium of Pleurotus ostreatus identified by submerged and solid-state cultures. Appl Biochem Biotechnol 102–103:463–469
13. Kang J, Chen RY, Yu DQ (2006) Five new diels-alder type adducts from the stem and root bark of *Morus mongolica*. Planta Med 72:52–59
14. Kim YO, Han SB, Lee LW, Ahn HJ, Jung Yoon YD, JK Kim HM, Shina CS (2005) Immuno-stimulating effect of the endopolysaccharide produced by submerged culture of *Inonotus obliquus*. Life Sci 77:2438–2456
15. Kim YO, Park HW, Kim JH, Lee JY, Moon SH, Shin CS (2006) Anti-cancer effect and structural characterization of endopolysaccharide from cultivated mycelia of *Inonotus obliquus*. Life Sci 79:72–80
16. Kukulianskaia TA, Kurchenko NV, Kurchenko VP, Babitskaia VG (2002) Physicochemical properties of melanins produced by *Inonotus obliquus* ("chagi") in the nature and the cultivated fungus. Prikl Biokhim Mikrobiol 38:68–72
17. Lee SH, Kim BG, Kim KJ, Lee JS, Yun DW, Hahn JH, Kim GH, Lee KH, Suh DS, Kwon ST, Lee CS, Yoo YB (2002) Comparative analysis of sequences expressed during the liquid–cultured mycelia and fruit body stages of *Pleurotus ostreatus*. Fungal Genet Biol 35:115–134
18. Li SP, Su ZR, Dong TT, Tsim KW (2002) The fruiting body and its caterpillar host of Cordyceps sinensis show close resemblance in main constituents and anti-oxidation activity. Fungal Genet Biol 9:319–324
19. Lindequist U, Julich WD (2005) The pharmacological potential of mushrooms. ECAM 2:285–299
20. Park YK, Lee HB, Jeon EJ, Jung HS, Kang MH (2004) Chaga mushroom extract inhibits oxidative DNA damage in human lymphocytes as assessed by comet assay. Biofactors 21:109–112
21. Park YM, Won JH, Kim YH, Choi JW, Park HJ, Lee KT (2005) In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. J Ethnopharmacol 101:120–128
22. Park JR, Park JS, Jo EH, Hwang JW, Kim SJ, Ra JC, Aruoma OI, Lee YS, Kang KS (2006) Reversal of the TPA-induced inhibition of gap junctional intercellular communication by Chaga mushroom (*Inonotus obliquus*) extracts: effects on MAP kinases. Biofactors 27:147–155
23. Pegler DN (2001) Useful fungi of the world: amadou and chaga. Mycologist 15:153–154
24. Preethi KC, Kuttan G, Kuttan R (2006) Antitumour activity of *Ruta graveolens* extract. Asian Pac J Cancer Prev 7:439–443
25. Rzymowska J (1998) The effect of aqueous extracts from *Inonotus obliquus* on the mitotic index and enzyme activities. Boll Chim Farm 137:l3–15
26. Shin Y, Tamai Y, Terazawa M (2001) Chemical constituents of Inonotus obliquus. II. A new triterpene, 21,24-cyclopentalanosta-3β, 21,25-triol-8-ene from sclerotium. J Wood Sci 47:313–316
27. Smieoff N, Cunbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28:1057–1060
28. Stephane LG, Brunhilde D, Monique P, Dominique B, Philippe D (2006) Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. Agr Food Chem 54:9193–9200

29. Sutton DA, Thompson EH, Rinaldi MG, Iwen PC, Nakasone KK, Jung HS, Rosenblatt HM, Paul ME (2005) Identification and first report of *Inonotus* (*Phellinus*) *tropicalis* as an etiologic agent in a patient with chronic granulomatous disease. J Clin Microbiol 43:982–987
30. Wang QL, Lin M, Liu GT (2001) Antioxidative activity of natural isorhapontigenin. Jpn J Pharmacol 87:61–66
31. Wu WL, Chang WL, Chen CF (1991) Cytotoxic activities of tanshinones against human carcinoma cell lines. Am J Chin Med 19:207–216
32. Xu J, Rao JR, Millar BC, Eleorn JS, Evans J, Barr JG, Moore JE (2002) Improved molecular identification of Thermoactinomyces spp. associated with mushroom worker's lung by 16S rDNA sequence typing. J Med Microbiol 1:1117–1127
33. Yoshiyuki K, Masahiko T, Kimiye B (2002) Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: mechanism of action and isolation of an active substance. Anticancer Res 22:3309–3318
34. Zjawiony JK (2004) Biologically active compounds from Aphyllophorales (Polypore) fungi. J Nat Prod 67:300–310