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Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*

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ABSTRACT

The medicinal mushroom *Inonotus obliquus* has been used as a folk remedy for a long time in Russia and East-European countries to treat gastrointestinal cancer, cardiovascular disease and diabetes. In our study, a water-soluble polysaccharide (ISP2a) was successfully purified from *I. obliquus* by DEAE-Sepharose CL-6B and Sepharose CL-6B column chromatography. *In vivo* ISP2a had not only shown antitumor activity, but also could significantly enhance the immune response of tumor-bearing mice. In addition, ISP2a significantly enhanced the lymphocyte proliferation and increased the production of TNF-α. Results of these studies demonstrated that ISP2a had a potential application as natural antitumor agent with immunomodulatory activity.

1. Introduction

Recently polysaccharides extracted from plants and fungi have drawn more attention of researchers and consumers due to their relatively low toxicity and obvious antitumor activities (Wang et al., 1997; Wasser, 2003). Therefore, discovery and evaluation of polysaccharides with antitumor and immunostimulating properties has emerged as one of important research fields in chemistry and biology (Zhang & Huang, 2005).

Edible and medicinal mushrooms have an established useage history in the human diet and traditional therapies (Wasser, 2003). The medicinal mushrooms *Inonotus obliquus* (*I. obliquus*) is a white rot fungus that belongs to the family Hymenochaetaceae of Basidiomycetes. *I. obliquus*, which is mainly distributed at latitudes of 45–50°N. *I. obliquus* has been used as a folk remedy to treat gastrointestinal cancer, cardiovascular disease and diabetes for a long time in Russia and East-European countries (Cui, Kim, & Park, 2005; Sun, Ao, & Lu, 2008). Researches revealed that a decoction of fungal sclerotia did not show toxic effects and has been used in treatment of cancers and digestive system diseases (Kim et al., 2006; Saar, 1991). Although the low-molecular-weight chemical constituents from *I. obliquus* have been investigated by several research groups (Kim et al., 2006; Solomon & Alexander, 1999; Tanaka, Toyoshima, & Yamada, 2011), the high-molecular-weight polysaccharides with significant activity have not been sufficiently investigated. The detailed investigation on the composition and activity of polysaccharides from *I. obliquus* was particularly necessary to broaden its applications in pharmaceutical industries and functional foods. Therefore, the aim of the present study was mainly to investigate the chemical properties of a water-soluble polysaccharide from *I. Obliquus* after its extraction and purification by ion exchange chromatography and gel filtration chromatography. Furthermore, the antitumor and immunomodulatory activity on the immune response in tumor bearing mice were also explored.

2. Materials and methods

2.1. Materials and equipments

I. obliquus was obtained from Yabuli Co. Ltd. (Heilongjiang, China). DEAE-Sepharose CL-6B and Sepharose CL-6B were purchased from Pharmacia Chemical Co. Human gastric carcinoma SGC-7901 cells were obtained from Shanghai Institute of Cell Biology (China). Medium RPMI1640, dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Life Technologies, Inc., USA). 5-Fluorouracil (5-Fu) was purchased from Jiangsu Hengrui pharmaceutics Co. (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co., USA. All

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other chemicals and solvents were analytical grade and used without further purification.

2.2. Extraction, isolation and purification of polysaccharides

Ground *I. obliquus* samples were pretreated with 95% ethanol and extracted with distilled water at 80 °C according to previous procedure (Li, Fan, & Ding, 2011). Crude water-soluble polysaccharides from *I. obliquus* (CISP) were obtained.

CISP was dissolved in 0.1 M phosphate buffer (pH 6.8) and filtered through 0.45 μm Millipore filter, and then the solution was subjected to DEAE-Sepharose CL-6B column chromatography (2.6 cm × 37 cm), which was equilibrated with 0.1 M phosphate buffer (pH 6.8) at room temperature. The column was first eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1.00 mL/min. The polysaccharides bound to the gel were eluted by increasing the ionic strength of the buffer. A linear gradient from 0 to 2 M potassium chloride in 0.1 M phosphate buffer (pH 6.8) was applied to the column. After the detection of elutes at 280 nm for protein and 490 nm for carbohydrate, the main fraction were fractionated by size-exclusion chromatography on a Sepharose CL-6B column (2.6 cm × 180 cm) eluted with 0.02 M NaCl at a flow rate of 0.5 mL/min. The salts were removed by dialysis and the purified polysaccharides (ISP2a) were obtained after concentration.

2.3. Molecular weight and composition analysis of ISP2a

Gas chromatography (GC) was used for identification and quantification of the monosaccharide (Li, Shan, Liu, Fan, & Ai, 2011). The homogeneity and average molecular weight of ISP2a were determined by high performance size-exclusion chromatography (HPSEC) using a Waters HPLC system, including two serially linked Ultrahydrogel™ Linear (Ø 7.8 mm × 300 mm ID) columns, a Waters 2410 differential refractive index detector and an on-line degaser. The mobile phase was 0.1 M $NaNO_3$. The molecular weights of the polysaccharide fractions were determined by comparison with retention times of Dextran T-series standards (MW: 6100, 16,500, 26,290, 40,000, 84,000 and 158,000).

2.4. Biological assays

2.4.1. Cell lines and culture

Human gastric carcinoma SGC-7901 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/L) at 37 °C in a humidified atmosphere with 5% CO_2. The culture was passaged every 2 or 3 days. Finally, cell concentration was 3×10^7 cells/mL.

2.4.2. Assay of inhibition of tumor cell proliferation in vitro

The inhibition effects of ISP2a on SGC-7901 cells (3×10^7 cells/mL) proliferation were determined *in vitro* using the colorimetric MTT assay (Chen, Huang, Gu, Tang, & Li, 2010). The dosages of ISP2a were 25, 50, 100, 150 and 200 μg/mL, respectively. The absorbance was measured at 570 nm by a 96-well microplate ELISA reader. All< determinations were done in triplicate. Inhibition ratio of tumor cell proliferation was calculated according to the formula below:

$$\text{Inhibition ratio } (\%) = \left(1 - \frac{\text{OD}}{\text{OD}_0}\right) \times 100$$

where OD and OD_0 are the absorbance of treated cells and untreated cells.

2.4.3. Assay of antitumor activities in vivo

All nude mice (3–5 weeks old, weighing 20 ± 2 g, male-to-female ratio of 1:1) were purchased from the Animal Center of Academy of Military Medical Science (Beijing, China). Firstly, the nude mice were injected subcutaneously into the right groin with 0.2 mL SGC-7901 cells (3×10^7 cells/mL) and randomly divided into four groups with 10 mice per group. After 24 h of tumor implantation, the tumor bearing mice were orally treated with the different concentration of ISP2a at 50, 75 and 100 mg/kg everyday for 10 days, and normal saline (10 mL/kg day) used as negative control, 5-fluorouracil (5-Fu) dissolved in 0.9% aqueous NaCl (20 mg/kg day) used as positive control. Then the mice were sacrificed by cervical dislocation. The spleen, thymus and tumor were dissected and weighted. The antitumor activity of ISP2a *in vivo* was expressed as an inhibitory rate calculated as:

$$\text{Inhibition ratio } (\%) = \left[\frac{A - B}{A}\right] \times 100$$

where *A* is the average tumor weight of untreated control group; *B* is the average tumor weight of treated groups. This study was repeated three times.

Blood was collected without the anticoagulant and serum was immediately separated by centrifugation at 3000 rpm at room temperature for 10 min. The concentration of TNF-α in serum of the treated mice was measured by enzyme-linked immunosorbent assay (ELISA) at 450 nm.

2.4.4. Spleen lymphocyte proliferation assay

The cell proliferation was assessed by using MTT-based colorimetric assay (Mosmann, 1983). Briefly, spleen lymphocytes (100 μL) were set up in 96-well plates (1×10^6 cells/mL), then 20 μL polysaccharides solution (25, 50, 100, 200, 400 μg/mL) and 80 μL RPMI 1640 medium was added with or without Con A (final concentration of 5 μg/mL). The control was added same volume of RPMI-1640 solution. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 44 h, MTT (20 μL, 5 mg/mL) was added to each well and the plates were incubated for another 4 h. The absorbance of spleen lymphocytes cells in each well were measured at 570 nm by ELISA reader (DG-3022A) according the method of Sun, Wang, et al. (2008).

2.4.5. Macrophage phagocytosis assay

All nude mice were injected intraperitoneally with sterile thioglycollate medium (2 mL) daily for 3 days. Macrophages were obtained by peritoneal lavage and were cultured in RPMI-1640 supplemented with 10% fetal calf serum in wells of 96-well plates for 2 h at 37 °C in a 5% CO_2 cell incubator. Non-adherent cells were removed by washing the wells with warm phosphate buffered saline (0.01 M PBS, pH 7.4) and the remaining cells were incubated at 37 °C under various concentrations of ISP2a ranging from 25 to 400 μg/mL. After 24 h incubation with MTT solution, the absorbance of each well was read at 570 nm using an ELISA reader.

2.4.6. Assay of TNF-secretion

After the mice were sacrificed, peritoneal macrophages (1×10^7 cells/mL) was collected and cultured with ISP2a at the concentration from 25 to 400 μg/mL in wells of 48-well plates for 24 h. The supernatants were collected for detection of TNF-α levels using commercial ELISA kits at 450 nm on an automatic ELISA plate reader. LPS (25 μg/mL) was used as a positive control.

2.5. Statistical analysis

All experiments were performed at least in duplicate, and analyses of all samples were run in triplicate and averaged. Statistical analysis involved use of the Statistical Analysis Systems (SAS, version 8.1) software package. The results shown were presented as means of three determinations ± SD (standard deviation). The

results obtained were analyzed using one-way analysis of variance (ANOVA) for mean differences among the samples. p-Values of <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Composition and molecular weight of polysaccharides

CISP was purified into four peaks by anion-exchange chromatography (DEAE-Sepharose CL-6B), which were ISP1 recovered from the NaAc-buffer eluate, and ISP2, ISP3 and ISP4 recovered from potassium chloride eluate. After the detection of lymphocytes proliferation at the concentration of 100 μg/mL, the ratio of splenocytes proliferation of ISP1, ISP2, ISP3 and ISP4 reached to 9.3%, 101.2%, 43.5% and 75.4%, respectively. ISP2 significantly enhanced the splenocyte proliferation. Therefore, it was further purified through Sepharose CL-6B column. Two water-soluble polysaccharides of ISP2a and ISP2b were obtained and ISP2a is a homogeneous polysaccharide through the analysis on high performance size-exclusion chromatography.

ISP2a had negative responses to the Bradford test and no absorption at 280 nm, indicating the absence of protein. The negative results of Fehling's and iodine-potassium iodide tests showed ISP2a did not contain reducing sugar and it did not belong to starch-type polysaccharide (Yang, Zhao, & Lv, 2008). The monosaccharide composition of ISP2a was determined by the trifluoracetic acid hydrolysis method. ISP2a was composed of rhamnose, mannose and glucose in molar ratios of 1.0:2.3:1.7. The monosaccharide composition was similar to previous report that polysaccharides from *I. obliquus* were mainly composed of glucose and mannose (Kim et al., 2006). Furthermore, total uronic acid content was measured by carbazole–sulfuric acid method using glucuronic acid as the standard. The results showed that the good linearity was obtained by regression analysis between A (absorbance) and C (μg). The uronic acid contents in ISP2a were 7.5%. The average molecular weight of ISP2a was 9.3×10^4 Da, according to the calibration curve with standard dextrans.

3.2. Inhibition of tumor cell proliferation in vitro

Cancer is a formidable problem for people. The great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells (Borchers, Stern, Hackman, Keen, & Gershwin, 1999). Therefore, it is very important to investigate novel antitumor substances with little toxicity to host. Recently, polysaccharides extracted from plants, fungi, algae and animals have been proven to be fewer side effects and possess a wide range of biological functions such as antitumor, antioxidant, immunomodulation and anti-tussive properties (Ananthi et al., 2010; Cheng et al., 2010). In this investigation, antitumor activity of ISP2a was evaluated *in vivo* and *in vitro*.

In vitro antitumor activities of ISP2a against SGC-7901 cells were investigated, and the results were shown in Fig. 1. The inhibition ratio was little enhanced with increasing of concentration of ISP2a. Even at a high concentration of 200 μg/mL, the inhibition ratio in suppressing the growth of SGC-7901 tumor cells was lower than 6%. The results showed ISP2a exhibited no significant antitumor activities and the growth of SGC-7901 cells was not directly affected by ISP2a treatment *in vitro*. Although these results were different from the other known polysaccharides from *Chlorella pyrenoidosa* and *Ornithogalum caudatum* (Chen, Meng, et al., 2010; Sheng et al., 2007), all of which could directly inhibit the proliferation of cancer *in vitro*. Our result is in agreement with many others findings. Kim et al. (2006) reported the endo-polysaccharide from cultivated mycelia of *I. obliquus* does not inhibit the growth of cancer cells directly *in vitro*. Peng, Zhang, Zeng, and Xu (2003) reported no obvious antitumor activities were found in extracellular polysaccharide of *Ganoderma tsugae* mycelium *in vitro*.

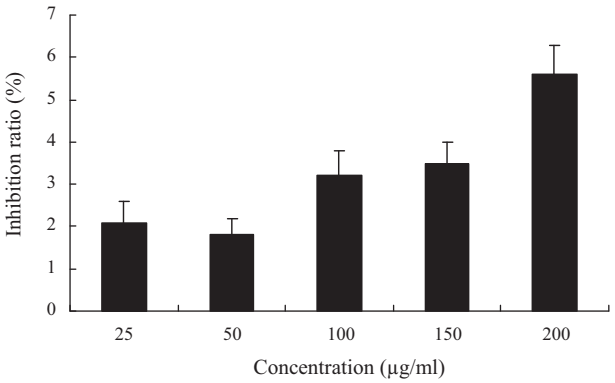

Fig. 1. Effect of ISP2a on SGC-7901 cells proliferation *in vitro*. (The results were expressed as mean values ± SD.)

3.3. Antitumor activities of ISP2a in vivo

The results of antitumor activities of ISP2a against SGC-7901 tumor-bearing nude mice *in vivo* are summarized in Table 1. After 10 days treatment, the growth of transplantable SGC-7901 in mice was significantly inhibited by ISP2a compared with the negative control ($p < 0.05$), with the inhibitory rate being 39.02%, 48.24% and 57.45% at the ISP2a concentration of 50, 75 and 100 mg/kg, respectively. This result correlated to the findings of Chen, Huang, et al. (2010) and they found that polysaccharides from *I. obliquus* had the higher antitumor activities *in vivo*. Simultaneously the immune organ weights of mice treated with ISP2a were also increased significantly. The relative spleen and thymus weights were obviously enhanced by ISP2a treatment than those of the negative group ($p < 0.05$), which was well in agreement with the antitumor activity. At present, the proposed mechanisms which polysaccharides exert antitumor effect include cancer-preventing, immuno-enhancing and direct tumor inhibition. The present results showed that ISP2a significantly increased the concentration of TNF-α in serum of mice, which indicated that the ISP2a may indirectly play the role of antitumors activity through the releases of effective molecules TNF-α. However, the antitumor mechanisms of ISP2a remain unclear, which are demanded for further study in the subsequent research.

3.4. Spleen lymphocyte proliferation

The immune system plays an important role in antitumor defense. Splenocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. T and B lymphocytes are two important classes of immunologically active cells. The former is mainly responsible for cellular immunity, and the latter is the only cell capable of producing antibodies. Some investigators have reported that polysaccharides isolated from plants or fungi are immunoactivator. For example, a red algae polysaccharide designated λ-carrageenan can inhibit tumor growth via the activation of natural killer (NK) cells and promote lymphocyte proliferation (Zhou et al., 2004). The funoran polysaccharide from *Gloiopeltis tenax* has been shown the ability of increasing spleen weight and augmenting T-helper, T-cytotoxic, and NK cells on tumor-bearing mice (Ren, Wang, Noda, Amano, & Ogawa, 1995). Many reports suggested that antitumor activity of the polysaccharides were also mediated through enhancing the immune response (Cho & Leung, 2007; Schepetkin & Quinn, 2006). The immunologic action of polysaccharides may begin with the

Table 1
Antitumor effects of ISP2a on SGC-7901 tumor-bearing nude mice *in vivo*.[a]

Group	Dose (mg/kg)	Inhibition ratio (%)	Relative spleen weight (mg/g)	Relative thymus weight (mg/g)	TNF-α (pg/mL)
Control	–	–	6.56 ± 0.27b	2.11 ± 0.13c	209.23 ± 25.12c
5-Fu	20	53.12	7.79 ± 0.32a	2.85 ± 0.29a	337.16 ± 17.14a
ISP2a	50	39.02	6.63 ± 0.25b	2.31 ± 0.18b	267.32 ± 26.22bc
ISP2a	75	48.24	7.38 ± 0.37ab	2.61 ± 0.13ab	295.28 ± 15.36b
ISP2a	100	57.45	7.95 ± 0.22a	3.03 ± 0.21a	362.82 ± 30.39a

[a] Relative thymus weight was measured in the ratio of thymus weight (mg) to body weight (g). Relative spleen weight was measured in the ratio of the spleen weight (mg) to body weight (g). Each value is expressed as means ± standard deviation ($n = 3$). Means with different letters within a column are significantly different by Bonferroni t-test ($p < 0.05$).

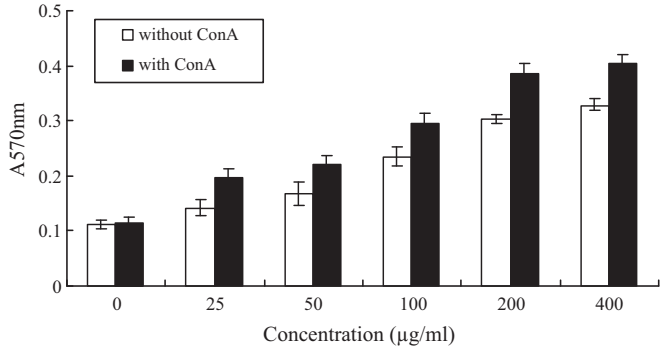

Fig. 2. Effect of ISP2a on lymphocyte proliferation. (The results were expressed as mean values ± SD.)

activation of effector cells such as lymphocytes and macrophages. Therefore, the effect of ISP2a on peritoneal macrophages and spleen lymphocyte were investigated in this paper. The effect of ISP2a on lymphocyte proliferation was shown in Fig. 2. All of the tested dosages with ISP2a could significantly enhance splenocyte proliferation as well as ConA-induced splenocyte proliferation ($p < 0.05$). However, ISP2a with ConA had stronger splenocytes proliferation activity than that without ConA. The higher the dosage of ISP2a, the higher increase in lymphocytes proliferation activity was observed, but the proliferation activity of ISP2a to spleen cells did not respond in the dose-dependent manner. The ratio of splenocytes proliferation reached to 109.8% at the ISP2a concentration of 100 μg/mL, while it was 170.5% and 192.8% at the concentration of 200 and 400 μg/mL, respectively. ISP2a at the low concentration strongly increased proliferation of splenocytes, while ISP2a at the high concentration weakly increased splenocytes proliferation.

3.5. Macrophage phagocytosis

Macrophage is the most important professional phagocyte and it plays an essential and pivotal role in host defense against any type of invading cells including tumor cells (Katsiari, Liossis, & Sfikakis, 2010). ISP2a was studied for its influence on peritoneal macrophage phagocytosis, and the effect of ISP2a on phagocytosis of macrophages was shown in Fig. 3. Compared with the control group, ISP2a at the concentration of 100, 200 and 400 μg/mL significantly enhanced the proliferation of peritoneal macrophages, but no significant improvement was observed between the dosages of 200 and 400 μg/mL. This proliferate activity of peritoneal macrophages had also been demonstrated by Li, Shan, Liu, Fan, and Ai (2011) who found the polysaccharides from *Zizyphus Jujuba cv. Jinsixiaozao* could activate mice macrophages.

3.6. TNF-α secretion

TNF-α plays an important role in tumoricidal and immune response and tumor cell elimination is known to be mediated in part by TNF-α. TNF-α can be produced by activated peritoneal

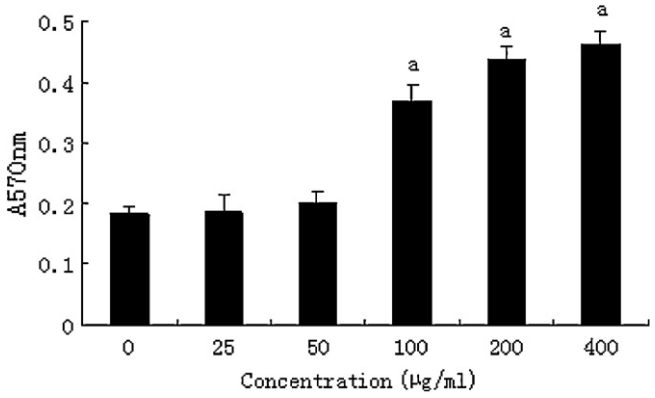

Fig. 3. Effect of ISP2a on phagocytosis of macrophages. (The results were expressed as mean values ± SD, and a indicates a significant difference compared with control at the level of 0.01.)

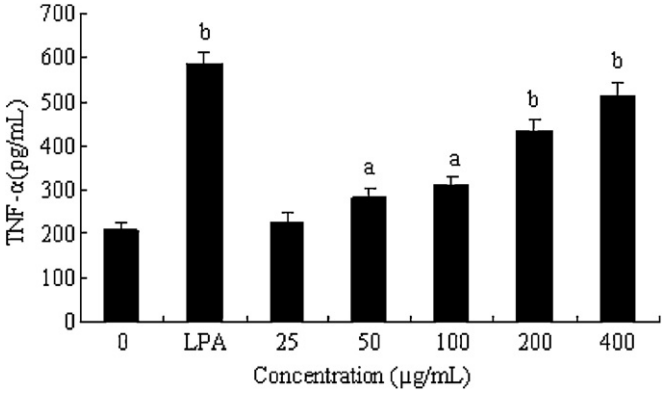

Fig. 4. Effect of ISP2a concentration on secretion of TNF-α by macrophages. (The results were expressed as mean values ± SD, a and b indicate the significant difference at the level of 0.05 and 0.01 compared with control.)

macrophages. The effect of ISP2a on TNF-α production was shown in Fig. 4. Compared with the control, ISP2a at the concentration of 50, 100, 200 and 400 μg/mL significantly enhanced the production of TNF-α. There exists a dose-dependent relationship with the highest concentration giving the strongest TNF-α production ($R^2 = 0.932$). The modulation of TNF-a production may contribute to part of the therapeutic effects of ISP2a.

4. Conclusions

According to the results stated above, ISP2a were successfully purified from *I. obliquus* by DEAE-Sepharose CL-6B and Sepharose CL-6B column chromatography. *In vitro* ISP2a exhibited no significant antitumor activities. However, *in vivo* ISP2a had not only shown antitumor activity, but also could significantly enhance the immune response of tumor-bearing mice. ISP2a could significantly enhance the lymphocyte proliferation and improve the ability of

ConA-induced lymphocyte proliferation. At the same time, ISP2a could mediate phagocytosis and increase the production of TNF-α compared with control. The results indicated that the ISP2a may indirectly play the role of antitumors activity through improving immunologic function of tumor-bearing mice. However, the antitumor mechanisms of ISP2a remain unclear, which are demanded to study further in the subsequent research.

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