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Characterisation and immunostimulatory activity of an α-(1→6)-D-glucan from the cultured *Armillariella tabescens* mycelia

Xia Luo [a,b], Xiaoyan Xu [b], Mengyao Yu [a,b], Zhirong Yang [a], Linyong Zheng [a,c,*]

[a] College of Life Sciences, Sichuan University, 610064 Sichuan, China
[b] Sichuan Institute of Chinese Material Medica, Chengdu, 610041 Sichuan, China
[c] Soil and Fertilizer Institute, Sichuan Academy of agricultural Sciences, 610066 Sichuan, China

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ABSTRACT

IPS-B2, an intracellular polysaccharide with anti-tumor effect, was isolated from cultured *Armillariella tabescens* mycelia by hot water extraction, anion-exchange and gel chromatography. Based on the results of paper chromatography (PC), gas chromatography (GC), infra-red (IR) spectroscopy and ^{13}C NMR, IPS-B2 was characterized as an α-(1→6)-D-glucan with a molecular weight of 49.5 kDa. The effects of IPS-B2 on murine peritoneal macrophages were further investigated. The results demonstrated that IPS-B2 induced nitric oxide (NO) and cytokines (TNF-α, IL-1β and IL-6) production in macrophages. The outcomes of real-time reverse transcription-polymerase chain reaction (RT-PCR) proved that the transcribing level of inducible NO synthase (iNOS), TNF-α, IL-1β and IL-6 mRNA in the peritoneal macrophages have been augmented by IPS-B2. These data suggest that the anti-tumor activity of the polysaccharide from *A. tabescens* may due to activation of macrophage.

1. Introduction

Armillariella tabescens (Scop.ex Fr.) Sing is one kind of edible mushroom which has been consumed as tasted food and health nutritional supplement for years. Armillarisin purified from *A. tabescens* has been used to cure liver disorder in traditional oriental remedy for decades (Yang, 2004; Zhu, Luan, & Shi, 1992. It was reported that the polysaccharides extracted from *A. tabescens* could suppress tumor proliferation and prolong lifespan of tumor-bearing mice (Kiho, Shiose, Nagai, Sakushima, & Ukai, 1992a, 1992b). And in our previous study, 18 mg/kg crude polysaccharides from *A. tabescens* could inhibit the proliferation of Sarcoma 180 (S180) tumor cells *in vivo* to the extent of 44.2% (Gao, Bai, & Luo, 2005). Further investigation showed that the anti-tumor activity of the crude polysaccharides was based on their immunomodulatory function. It was found that the crude polysaccharides could increase spleen and thymus index, stimulate the proliferation ability of lymphocyte and enhance the activity of NK cells in S180-bearing mice significantly (Zhao, 2006).

Macrophages play a crucial role in the immune system. Activated macrophages are considered as the pivotal immunocytes of host defense which against tumor growth (Fidler & Kleinerman, 1993). The tumoricidal activity of macrophages is performed mainly through NO and other cytokines (TNF-α, IL-12, etc.) (Flick & Gifford, 1984). Especially, NO is considered to be a central molecule in the regulation of tumor immune (Klostergaard, 1993; Paulnock, 1992).

The present investigation elucidates the isolation and characterization of an intracellular polysaccharide with anti-tumor effect from cultured *A. tabescens* mycelia and its effects on the peritoneal macrophages (PM).

2. Materials and methods

2.1. Preparation of polysaccharide

A. tabescens (No. 5.92, purchased from China General Microbe Conservation Center) were inoculated into 30 L culture media (3 g/L glucose, 7 g/L sucrose, 3 g/L yeast extract, 5 g/L peptone, 0.1 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, pH 6.4) and incubated under the conditions of 200 rpm at 28 °C for 5 days. The culture was taken and centrifuged at 3500 rpm for 30 min. The fungi pellets (Dry Weight: 58.26 g) were then extracted with distilled water 6000 mL at 90 °C every 6 h for three times. The supernatants were collected by centrifugation and concentrated in vacuo. A combination method of papain enzymolysis and Sevag was applied for deproteinating the concentrated solution (Alum & Gupta, 1985). The crude polysaccharide fraction (4.9 g) was obtained through precipitation with four volumes of ethanol and desiccation in vacuo. The precipitate was redissolved in distilled water and

* Corresponding author. Address: College of Life Sciences, Sichuan University, 610064 Sichuan, China. Tel: +86 28 85250783; fax: +86 28 85250783.
E-mail address: xuxiaoyan629@gmail.com (L. Zheng).

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applied to DEAE-Cellulose A52 anion-exchange chromatography column (2.6 cm × 30 cm, Whatmann), eluting at a flow rate of 2 mL/min successively with distilled water and a gradient of 0–0.5 mol/L NaCl. According to the total carbohydrate content quantified by the phenol-sulfuric acid method, the yielded fractions were combined. The second fraction (IPS-B) was further purified on a Sephacryl S-200 column (2.6 cm × 100 cm, Pharmacia Co.). The column was eluted with 0.1 mol/L NaCl at a flow rate of 24 mL/h. Three separated fractions were got. The second fraction was collected, dialyzed and lyophilized to obtain a white purified *A. tabescens* polysaccharide (named IPS-B2, 177 mg, 3.61% of the crude polysaccharide).

2.2. Analytical methods

Total carbohydrate content was determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), by using D-glucose as the standard.

The homogeneity and molecular weight of the IPS-B2 were evaluated and determined by the HPGPC method on a Waters (2487) HPLC system equipped with a TSK gel 4000 PW_{XL} column (7.8 mm × 300 mm) and a Waters (2414) Refractive Index Detector (RID). The T-series Dextran standards (T10, T20, T70, T110, T500 and T2000) were adopted in this step for calibrating the column.

Paper chromatography (PC) and gas chromatography (GC) were used for identification and quantification. PC was performed on Xinhua No. 3 paper in the following solvent system: 6:4:3 *n*-butanol-pyridine-H_2O. As an internal standard, total hydrolysis of polysaccharide containing *myo*-inositol was performed with 2 M Trifluoroacetic acid (TFA) at 110 °C for 2 h. The sugars in the hydrolysate were converted to their alditol aetates and analyzed by GC on a HP 6890 instrument with a DB-225 column (0.25 mm × 15 mm) at temperature program of 50–230 °C with a rate of 2 °C/min, helium carrier gas at 1.2 mL/min, flame ionization detection (FID) at 270 °C.

IR spectroscopy and ^{13}C NMR were used to characterize the structural features of IPS-B2. The IR spectrum of the IPS-B2 was done using a Nicolet 170SX FTIR. The test specimens of polysaccharide film were prepared by the KBr-disk method. For ^{13}C NMR, The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and dissolved in D_2O. The ^{13}C NMR spectra were recorded with an INOVA-500 spectrometer at room temperature. All the chemical shifts were in relative to Me_4Si.

2.3. Peritoneal macrophage preparation

BALB/c albino mice of 6- to 8-week-old were injected intraperitoneally (i.p.) with 1 mL of 3% thioglycollate (Sigma Chemical Co., St. Louis, MO, USA) (Kim et al., 2003). Four days after injection, mice were euthanized and peritoneal exudate cells were collected by lavage with 5 mL of sterile cold D-Hank's. The exudate cells were collected and cultured in 60 mm dishes with RPMI-1640 (Gibco) containing 10% heat-inactivated FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (RPMI-FBS). After 1 h incubation at 37 °C, the cultures were washed twice with RPMI-1640 to remove non-adherent cells and the adherent cells were collected by gently scraped. The viability of macrophages was assessed by trypan blue exclusion.

2.4. Macrophage stimulation by IPS-B2

Macrophages (1×10^6 cells/mL) were plated in a 24-well plate (Corning, NY). The cells were cultured in phenol red-free RPMI-FBS medium containing increasing concentrations of IPS-B2 from 5 to 200 μg/mL (1 mL per well), and 10 μg/mL LPS was added in another wells as positive control. And negative control cells were cultured in medium individually. The experiments were performed in triplicate. For nitric oxide and cytokines detection the cells were incubated at 37 °C for 48 h, while the cells were cultured for 6 h at same conditions for detecting the mRNA.

2.5. Nitric oxide determination

The amount of stable nitrite and 100 μL of culture supernatants were mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4). This mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was read on a Thermo multiskan ascent reader. The nitrite concentration was determined by extrapolation based on a standard sodium nitrite curve (Keller, Geiges, & Keist, 1990).

2.6. Cytokines determination

The amount of TNF-α, IL-1β, IL-6 of culture supernatants were determined by ELISA kit (purchased from R&D Systems) following the instruction of manufacturers.

2.7. Quantitative RT-PCR detection of related gene expression

The peritoneal macrophages were harvested after stimulated by various concentration of IPS-B2 for 4 h. The total cellular RNA was extracted using Trizol Reagent (Invitrogen, USA) and reverse-transcribed into cDNA using oligo $(dT)_{18}$ primers (Invitrogen, USA). Amplification of each target cDNA was performed in the icycler system (Bio-rad, USA). PCR products were quantified using SYBR Green I. β-actin was used as an endogenous control to normalize expression levels among samples. A standard curve of each primer set was generated using LPS-induced macrophage cDNA. The PCR primers chosen were shown in Table 1.

The relative expression abundance was calculated by the following formula:

$$\text{Relative expression abundance} = \frac{\text{mols of detected mRNA}}{\text{mols of } \beta\text{-actin mRNA}}$$

2.8. Statistical analysis

The data were expressed as means ± SD. The significance of difference was evaluated with one-way ANOVA, followed by Student's *t*-test to statistically identify differences between the control and treated groups. Significant differences were set at $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Chromatography of A. tabescens polysaccharides

The crude polysaccharides can be separated into six parts by gradient elution of DEAE-cellulose ion-exchange column, namely IPS-A, IPS-B, IPS-C, IPS-D, IPS-E and IPS-F (Fig. 1). These six fractions were tested their anti-tumor effect on Sarcoma 180 bearing mice and IPS-B showed maximal anti-tumor effect (S180 inhibitory rate 44.2%, $P < 0.01$), so it is selected for further purified. By gel permeation isolation using Sephacryl S-200, IPS-B was divided into three fractions (IPS-B1, IPS-B2 and IPS-B3) (Fig. 2). The result of *in vivo* anti-tumor test showed that only IPS-B2 pointed out the significant difference when comparing against control group (S180 inhibitory rate 48.5%, $P < 0.01$).

Table 1
The result of primer design

Gene	Antisense (5′-3′)	Sense (5′-3′)	Tm (°C)	Product Size (bp)
β-actin	GCTGTCCCTGTATGC CTCT	TTGATGTCACGCACG ATTT	55.4	222
IL-1β	GCCCATCCTCTGTGACTC	CTGCTTGTGAGGTGCTGA	52.0	434
IL-6	GCCTTCTTGGGACTGATGCTGG	CTCTGGCTTTGTCTTTCTTGTT	51.7	385
TNF-α	GCCTATGTCTCAGCCTCT	GGTTGACTTTCTCCTGGTAT	53.4	423
iNOS	GAGCGAGTTGTGGATTGTC	GGGAGGAGCTGATGGAGT	55.2	376

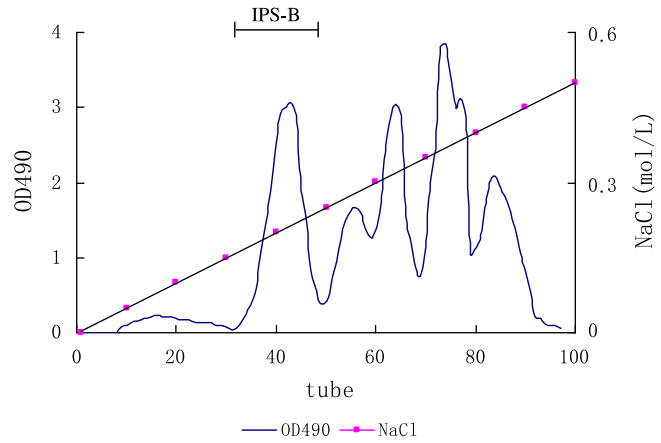

Fig. 1. DEAE-cellulose ion-exchange chromatography of crude polysaccharides. The column was eluted with a linear gradient of 0–0.5 M NaCl in the distilled water. (Flow rate = 2 mL/min, fraction size = 5 mL.) Carbohydrate was estimated from each fraction by phenol-sulfuric acid method.

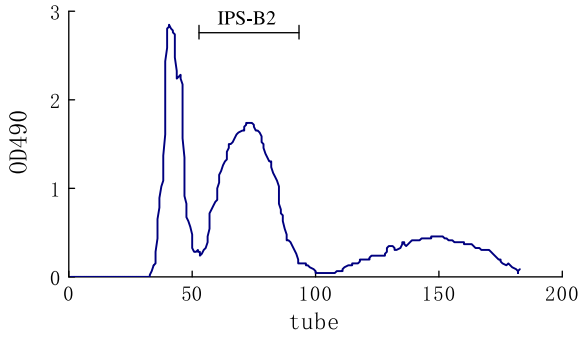

Fig. 2. Sephacryl S-200 gel filtration chromatography of IPS-B. The column was eluted with distilled water. (Flow rate = 24 mL/h, fraction size = 2 mL.) Carbohydrate was estimated from each fraction by phenol-sulfuric acid method.

3.2. Purification and structure characteristics of the polysaccharide

The yield of the crude water-soluble polysaccharide was 8.4% of the dried mycelia. The crude polysaccharide was loaded successively on DEAE-Cellulose A52 anion-exchange chromatography and the Sephacryl S-200 gel-permeation chromatography, which resulted in the complete isolation of IPS-B2 with a recovery of 0.3% contrast to the original dried mycelia. And IPS-B2 was found to be free of endotoxin by Limulus amoebocyte lysate gel-clot assay.

IPS-B2 was eluted as a symmetrical narrow peak on HPGPC. According to standard T-series Dextran HPGPC elution, the molecular weight was estimated to be 49.5 kDa. And the absence of absorbance at 280 nm indicated that the polysaccharide was protein free.

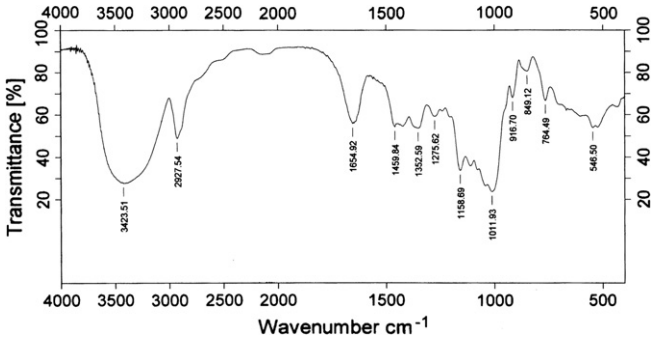

Fig. 3. IR spectrum of polysaccharide IPS-B2 isolated from *Armillariella tabescens* mycelia.

By the means of PC and GC, the quantitative determination of neutral monosaccharide component pattern indicated IPS-B2 only contains glucose, which means IPS-B2 is a glucan.

The IR spectrum of IPS-B2 (Fig. 3) showed a strong band at 3423.51 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2927.51 cm^{-1} was due to C–H stretching vibration. The band at 1654.92 cm^{-1} was due to the bound water (Park, 1971). The band at 849.12 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 916.70 cm^{-1} and 764.49 cm^{-1} were characteristic of D-pyranose-glucan. The three bands from 1100 cm^{-1} to 1010 cm^{-1} also demonstrated the existence of α-pyranose form of the glucosyl residue. The IR spectrum indicated the presence of α-glycosidic linkages in the IPS-B2 (Bao, Duan, Fang, & Fang, 2001; Tsumuraya & Misaki, 1979).

The structure of IPS-B2 comprised α-configuration of the D-glucosyl groups. It was conformed clearly by the presence of anomeric peaks in the region 100.216 ppm from ^{13}C NMR experiments (Fig. 4). At the same time, the ^{13}C NMR spectrum also indicated the (1→6)-α-D-Glcp in the IPS-B2. The resonances at 72.045, 72.701, 73.901, 75.911 and 68.070 ppm were assigned to protons of carbons C-2, C-3, C-4, C-5 and C-6 of glycosidic ring (Chi, Min, Yifan, & Weikang, 2006; Zhao, Kan, & Li, 2005).

Based on the above-mentioned results, it could be concluded that IPS-B2 was composed of a repeating unit with the possible structure as →6)-α-D-Glcp-(1→6)-[α-D-Glcp-(1→6)-]$_n$-α-D-Glcp-(1→.

3.3. IPS-B2 increases Nitric oxide production in PM

Nitric oxide is one of the major reactive oxygen species which produced by macrophage for destructing the targets. Since the final product of NO is either nitrite or nitrate, the sum of nitrite and nitrate level provides an indirect measurement of NO level. When murine macrophages were incubated with various concentrations of IPS-B2, the NO production increased in a dose-dependent manner. The NO increment plateaued at about 100 μg/mL of IPS-B2 (Fig. 5A). LPS, as a positive control, also induced NO production.

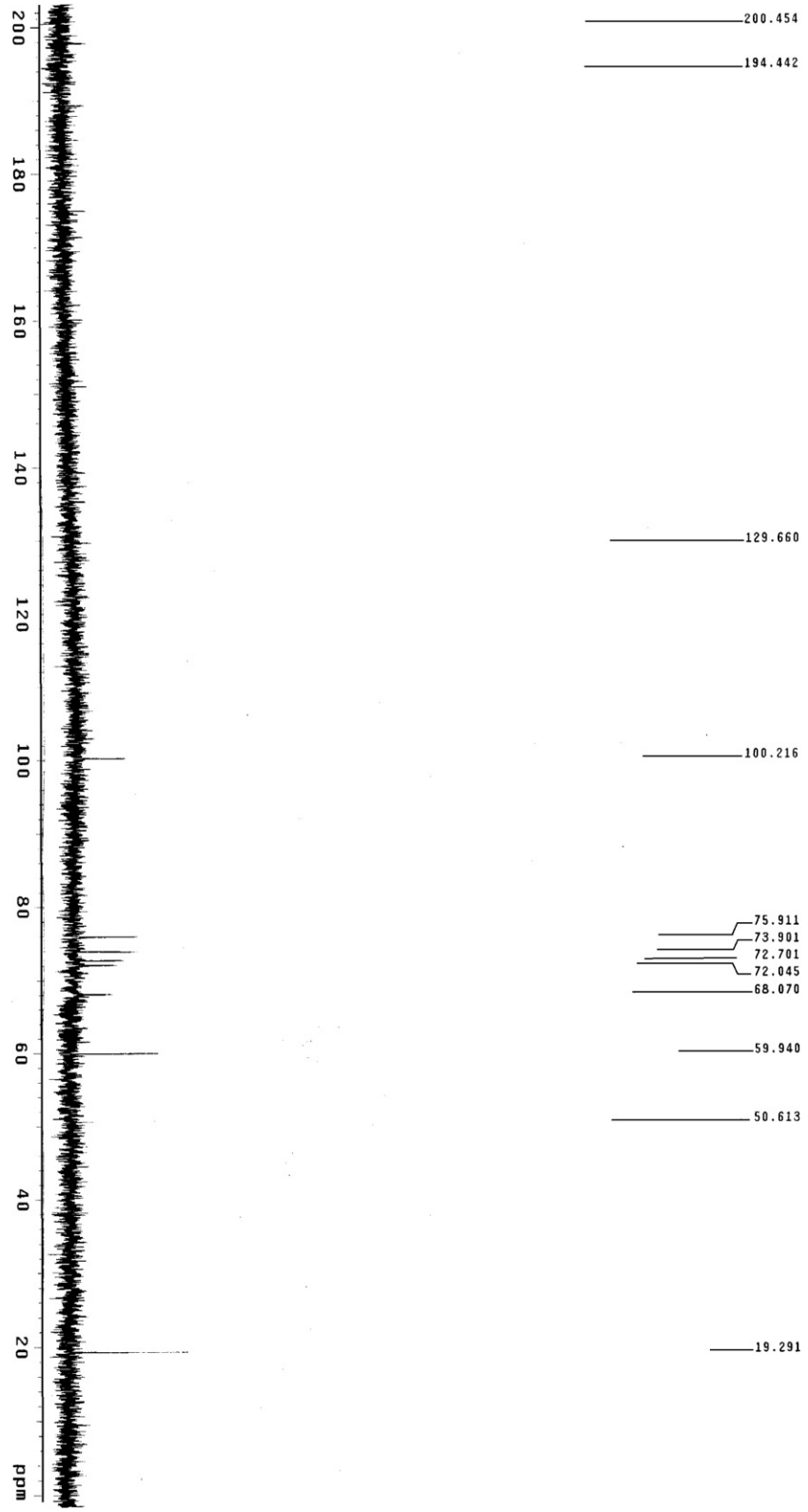

Fig. 4. ^{13}C NMR spectrum of polysaccharide IPS-B2 isolated from *Armillariella tabescens* mycelia.

3.4. IPS-B2 enhances cytokines productions in PM

Using the ELISA method, the level of IL-1β, IL-6, TNF-α, which secreted by IPS-B2-activated macrophages was compared with control. Obviously, the level of all three cytokines secreted by IPS-B2-stimulated macrophages was much higher than medium-treated macrophages. LPS of 10 μg/mL was the positive control. And the level of cytokines was induced by IPS-B2 treatment was similar to LPS at different concentration. It is noted that the IL-1β (Fig. 5B) production was increased in a dose-dependent manner,

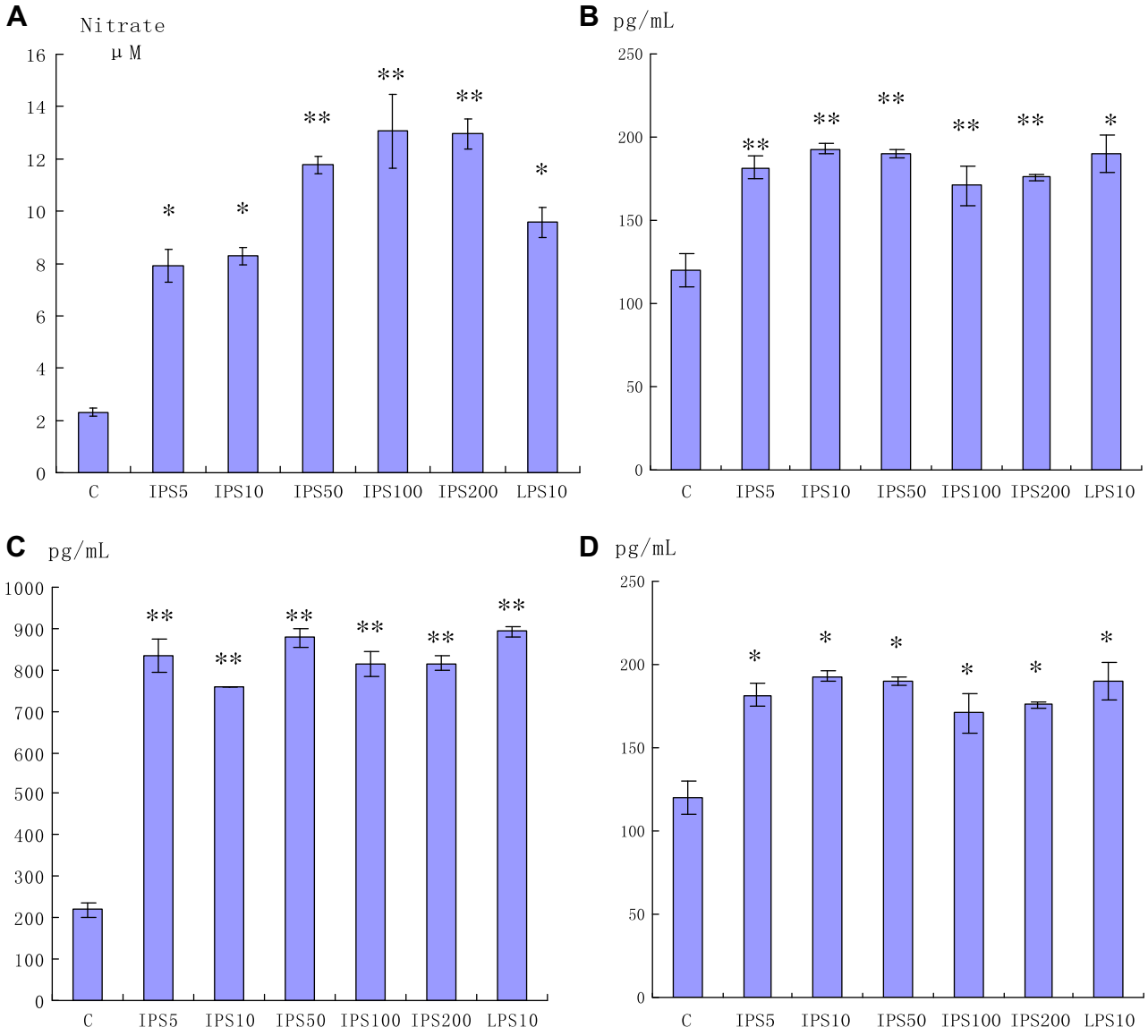

Fig. 5. Production of NO and cytokines in PM stimulated by IPS-B2. This panel shows effects of IPS-B2 on (A) NO (B) IL-1β (C) IL-6 (D) TNF-α production in PM stimulated with various concentrations (5, 10, 50, 100 or 200 μg/mL). Results were expressed as means ± SD of three separate experiments. Significantly different ($^{*}P < 0.05$, $^{**}P < 0.01$) from medium alone (C).

while the productions of IL-6 and TNF-α were stimulated at a high level when the concentration of IPS-B2 was only 5 μg/mL (Fig. 5C and D).

3.5. IPS-B2 stimulated the expression of TNF-α, IL-1β, IL-6 and iNOS mRNA

Quantitative RT-PCR results showed a significant increase in the level of TNF-α, IL-1β, IL-6 and iNOS mRNA in IPS-B2-treated peritoneal macrophages compared to those untreated. The positive control, LPS 10 μg/mL, also promoted the expression of these genes. The expression of all the genes studied in the untreated macrophage was little, but increased dramatically in a dose-dependent manner in the IPS-B2-treated cells (Fig. 6A–D).

4. Discussion

In published papers, most mushroom polysaccharides showed their anti-tumor activities based on the β-(1→3)-D-glycosidic linkage structures. However, there was no consensus could be made on β-glucan and anti-tumor activity (Zhao et al., 2005). Recently, several reports on α-glucans with anti-tumor activity had been published. The α-glucans exhibited the anti-tumor activity from *Tinospora cordifolia, Ramalina celastri*, *Aconitum carmichaeli* and *Ipomaea batatas* and could modulate the immune by stimulating the proliferation of lymphocyte, enhancing cytotoxic effect of the NK cell, augmenting the expression of such cytokines and so on. (Chi et al., 2006; Nair, Rodriguez, Ramachandran, & Alamo, 2004; Zhao et al., 2005). In this study, one water-soluble polysaccharide (IPS-B2) from cultured *A. tabescens* mycelia was purified and the structure was investigated. Results showed that IPS-B2 was a D-glucan containing α-(1→6)-linked to backbone. Previous pharmacological tests suggested that IPS-B2 could inhibit significantly the growth of Sarcoma 180 tumor *in vivo* (Zhao, 2006). But it could not suppress the proliferation of tumor cells *in vitro*. Therefore, it was predicted that the anti-tumor effect was exhibited in an indirect way.

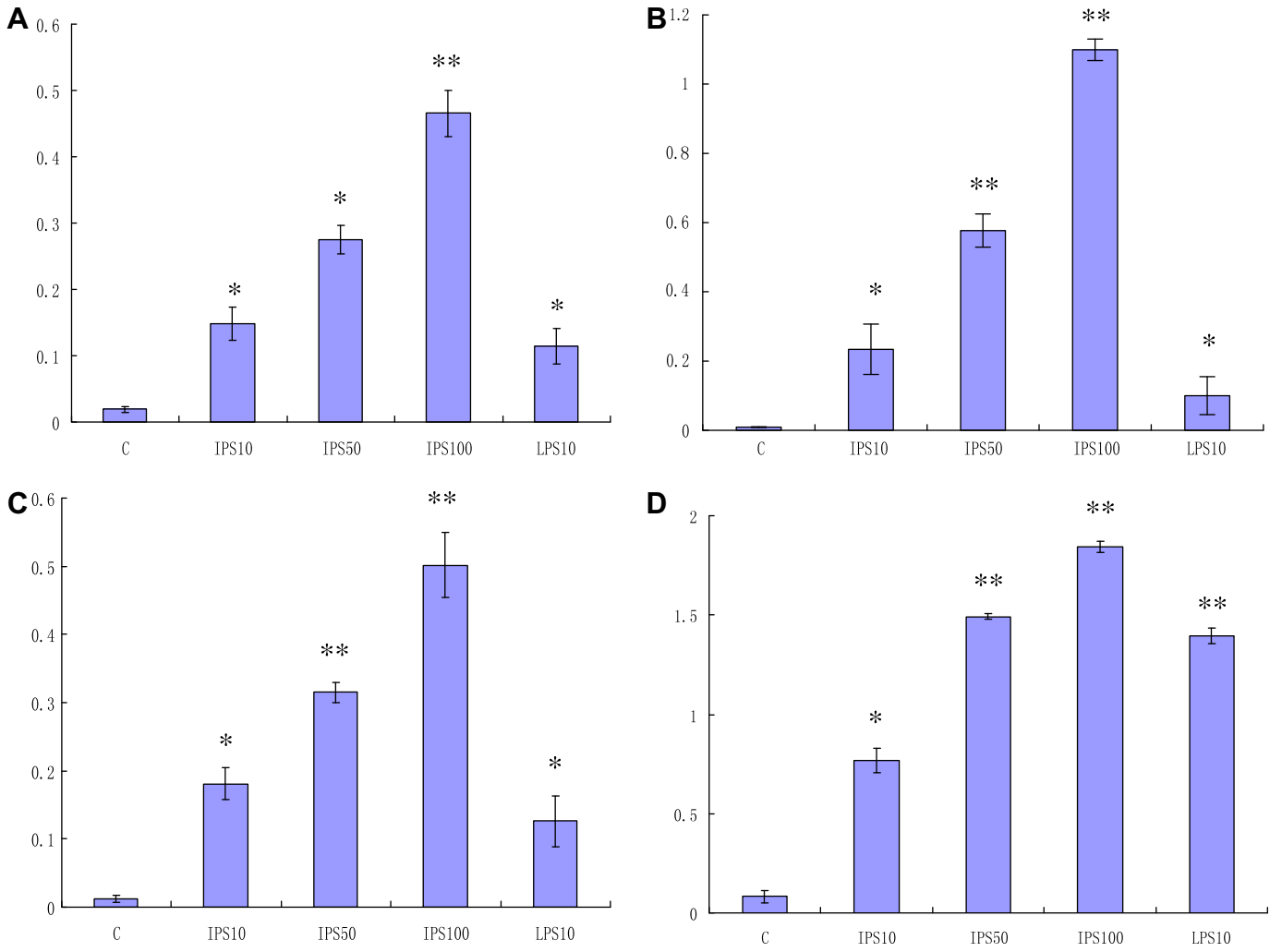

Fig. 6. Expression of IL-1β, IL-6, TNF-α and iNOS mRNA in PM stimulated by IPS-B2. This panel shows effects of IPS-B2 on (A) IL-1β (B) IL-6 (C) TNF-α (D) NO production in PM stimulated with various concentrations (10, 50 or 100 μg/mL). Results were expressed as means ± SD of three separate experiments. Significantly different ($^{*}P < 0.05$, $^{**}P < 0.01$) from medium alone (C).

The role of activated macrophages in the defense against tumor cells has been investigated extensively over the last decades (Fidler & Kleinerman, 1993; Flick & Gifford, 1984; Klostergaard, 1993). Accumulated evidence indicated that activated macrophages are able to recognize and lyse tumor cells including those that are resistant to cytostatic drugs. Therefore, macrophage activation can play a role in novel immunotherapeutic approaches to the treatment of cancer (Klimp, de Vries, Scherphof, & Daemen, 2002).

Macrophages can kill the Tumor cells either by macrophage-mediated tumor cytotoxicity or ADCC (Antibody-dependent cellular cytotoxicity). And both processes will end up in the manner of release of cytotoxic mediators including TNF-α, IL-1, NO and reactive oxygen intermediates or phagocytosis (Klimp et al., 2002).

TNF-α is one of most important mediators involved in tumor cell killing by the induction of multiple intracellular pathways, such as the generation of reactive oxygen intermediates in mitochondria preceding plasma membrane permeabilization (Goossens, Grooten, De Vos, & Fiers, 1995) and induction of iNOS-expression. Ultimately, these processes can lead to cell death. IPS-B2 could increase the secretion of TNF-α of macrophage and the expression of TNF-α mRNA *in vitro*. The toxic effects of NO and its derivatives on target cells are due to several mechanisms (Kroncke, Fehsel, & Kolb, 1995). Our results demonstrated that IPS-B2 could increase the releasing of NO and induced expression of iNOS gene to several folds higher in *vitro*. Taken together, it is reasonable to assume that the release of TNF-α and NO of macrophage which is activated by IPS-B2 may be the important mechanism of the anti-tumor effect of IPS-B2. However, further evidence is required.

It is well known that IL-1β performs as an important role in cancer immune-modulating. It is also shown that IL-1 has directed *in vitro* cytostatic and cytocidal effects (Lavnikova et al., 1997). IL-6 is also considered as a major immune and inflammatory mediator in cancer (Wang et al., 1997). This research indicated that the macrophage was induced to enhance the secretion and expression of the cytokines IL-1β and IL-6 by IPS-B2. These results points that these cytokines maybe involve in the tumoradical effect of IPS-B2.

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