



Research Article

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Separation, purification and identification of acidic polysaccharide fraction extracted from *Boletus edulis* and its influence on mouse lymphocyte proliferation *in vitro*

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ABSTRACT

To separate, purify and identify the acidic polysaccharide fraction in *Boletus edulis*, and to determine its influence on mouse lymphocyte proliferation. Crude polysaccharides were prepared from *Boletus edulis* by hot water extraction and ethanol precipitation. Acidic polysaccharide fractions were separated and purified from crude polysaccharides by DEAE-52 cellulose column, Sephadex G-100 and 0.1M NaCl solution elution. The relative molecular weight of the acidic polysaccharide fraction was determined by efficient liquid gel permeation method, the monosaccharide composition was analyzed by HPLC, and the influence of the acidic polysaccharide fraction on the activation and proliferation of mouse T and B cells was determined using the MTT assay. The purified acidic polysaccharide fraction isolated from *Boletus edulis* (APFB) with an average molecular weight (M_w) of 39668 was obtained. APFB was composed of eight monosaccharides, specifically mannose, xylose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose and fucose. Wherein, galacturonic acid had the highest content (32.1%). Concentrations between 5 $\mu\text{g/ml}$ and 640 $\mu\text{g/ml}$ APFB promoted proliferation *in vitro* of mouse spleen T cells stimulated by ConA for 72 h ($P < 0.05$). At APFB concentrations of 10 $\mu\text{g/ml}$ and more, APFB significantly enhanced proliferation *in vitro* of mouse spleen B cells stimulated by LPS for 72 h ($P < 0.05$). The acidic polysaccharide APFB separated and purified from *Boletus edulis* can enhance activation and proliferation *in vitro* of mouse spleen T and B cells. This suggests that APFB may be an effective immune regulator because it promotes effective humoral and cellular immune functions.

Keywords: *Boletus edulis*; acidic polysaccharides; purification; lymphocytes; proliferative activity

INTRODUCTION

Plant polysaccharides have a variety of biological activities, including regulating immune function, inhibiting tumor growth, resisting aging, viral infection and radiation, lowering blood fat and sugar levels, and protecting the liver. Plant polysaccharides have low toxic side effects on the body, and are therefore important components of many natural medicines and health care products. Plant polysaccharides have been widely used in functional foods, bio-medicine, and as bio-materials. For example, mushroom polysaccharides, *ganoderma lucidum* and *coriolus versicolor* have been widely used for clinical purposes [1-2]. The study of the extraction, separation and biological activity of acidic polysaccharides from plant polysaccharides is currently one of the most active fields in life science research [3-6].

Boletus edulis is a type of large fungus with ectotrophic mycorrhiza, which belongs to an important group of basidiomycetes that are found growing on trees in the wild. The fungus has a total of 89 genus and more than 1025 categories all over the world [7]. *Boletus edulis* is a large fleshy fungus, which contains 17 kinds of amino acids, including eight amino acids essential to the human body. Additionally, the proportion of the various amino acids contained in the fungus is similar to what the body needs. *Boletus edulis* is not only nutritious, but also has

medicinal value and has the reputation of being a 'longevity mushroom'. It is one of the raw materials in 'Shujin Pill', a Chinese medicine [8]. *Boletus edulis* polysaccharides extracted from *Boletus edulis* have been reported to improve immunity [9], resist tumors [10, 11], resist aging [8, 12] and lower blood fat [13, 14]. Current studies on *Boletus edulis* polysaccharides focus on crude polysaccharides or mixed polysaccharides and acidic polysaccharides are rarely studied [15, 16]. In this paper, we separate, purify and determine the molecular weight and monosaccharide composition of *Boletus edulis* acidic polysaccharides. We also investigate the proliferative effects of *Boletus edulis* acidic polysaccharides on mouse spleen T and B lymphocytes *in vitro*.

EXPERIMENTAL SECTION

***Boletus edulis* and Experimental Animals**

Dehydrated wild *Boletus edulis* product was purchased from China Zhejiang Lishui Mushroom Research Institute. SPF grade male BALB/c mice aged between 8 and 10 weeks old with weights ranging from 20 to 22 g were purchased from Shanghai Shien Experimental Animal Co., Ltd.

Experimental Reagents

LPS, ConA, MTT, dimethyl sulfoxide (DMSO) were purchased from Sigma (Sigma Co. Ltd., St Louis, MO, USA). RPMI-1640 medium was purchased from Gibco (Life Technologies, Grand Island, NY, USA). Mannose, xylose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, fucose, other monosaccharide control products and dextran T-series standard products (relative molecular weight of 10500, 43500, 43500, 76900 and 2000000 Da) were purchased from Sigma. DEAE-cellulose 52 and Sephadex G-100 were purchased from Pharmacia Corporation (Kalamazoo, MI, USA). Chromatographic column SEC-700 was purchased from U.S. Welch Co., Ltd.

Experiment Instruments

UV-2450 UV spectrophotometer, LC-20A efficient liquid chromatograph (Japan Shimadzu Co., Ltd.); RE-2000B rotary evaporator, ALphal-2 type vacuum freeze dryer (Germany CHRIST Co., Ltd.); Waters 515 type gel chromatography, Waters2410 differential refractive index detector (USA Milford Co., Ltd.).

Extraction of *Boletus edulis* Crude Polysaccharides

Dried *Boletus edulis* fruit (200 g) was pulverized and incubated at 90 °C in 2000 ml of water for 4h with two changes of water. The filtrate was concentrated to 500 ml. A 3-fold volume of 95% ethanol (1500 ml) was added and the filtrate placed statically overnight at 4 °C. The precipitate was collected by centrifugation at 3000 g for 15 min. The supernatant was discarded and the precipitate was washed with absolute ethyl alcohol, acetone and ether successively, then concentrated, filtered and lyophilized at 55 °C using a rotary evaporator. The product was bleached using 20% hydrogen peroxide, and deproteinized using the Sevage method [17], which was repeated in triplicate. It was then dissolved in water and dialyzed against deionized water for 72 h. The filtrate was centrifuged for sedimentation, frozen and dried under vacuum to yield *Boletus edulis* crude polysaccharides (CPB).

Separation and Purification of *Boletus edulis* Acidic Polysaccharides

The CPB product was dissolved in deionized water then added to an equilibrated diethylaminoethyl cellulose ion exchange column (60 mm × 300 mm). After the sample was loaded, it was eluted using deionized water to collect neutral polysaccharides(NPFB); then eluted using 0.1M NaCl solution for collecting acidic polysaccharides, with a flow rate of 1.0 ml/min. The phenol-sulfuric acid method [18] was adopted for tracing test, collection, dialyzation and lyophilization. The measured absorbance was the vertical axis, the tube number was abscissa, and the polysaccharide elution curve mapped. The sample was further purified by Sephadex G-100 (1.6 cm × 120 cm) gel column chromatography, and diluted with 0.1M NaCl solution. The flow rate was 0.5 ml/min. The main polysaccharide peak was collected. The filtrate was dialyzed against deionized water and lyophilized, yielding *Boletus edulis* acidic polysaccharides (APFB).

Ultraviolet Spectroscopy Analysis

The absorption spectra (wavelength range 200–400 nm) of a 1 mg/ml solution of APFB in deionized water was obtained.

Identification of Polysaccharide Purity and Molecular Weight

Acidic polysaccharides APFB purity and relative molecular weight was measured by gel permeation chromatography (GPC). 2 mg of APFB was dissolved in 1 ml deionized water and left overnight and then centrifuged at 2000 g for 15 min. 5 µl of the mixture was analyzed by Waters efficient liquid phase gel permeation chromatography (Waters ultrahydrogel column (7.8 mm × 300 mm), Waters2410 refractive index detector, mobile phase 0.2M phosphate buffer (pH 7.0), and flow rate 0.8 ml/min). Dextran T-series was used to prepare the standard curve, and the relative molecular weight was obtained from the standard curve according to the retention time of

polysaccharide samples under the same chromatographic conditions.

Monosaccharide Composition Analysis

APFB monosaccharides were analyzed by LC-20A efficient liquid phase chromatography system [19]. The analytical column was SEC-700 (4.6 mm × 700 mm). APFB was hydrolyzed into monosaccharide components with trifluoroacetic acid at 90 °C, and then analyzed at 250 nm using HPLC after the monosaccharides were marked by PMP.

Preparation of Mouse Spleen Lymphocyte Suspension

BALB/c mice were anesthetized and sacrificed. Spleens were aseptically removed and ground to prepare a single cell suspension. The cell suspension was centrifuged at 1800 g for 15 min and the supernatant discarded. Red blood cells were lysed using hemolysin. The suspension was centrifuged at 1500 g for 5 min and the cell pellet washed twice with cold PBS. The isolated cells were more than 95% viable by trypan blue staining and microscopic examination. The cell number was adjusted to a concentration of 5×10^6 cells/ml with complete GI-1640 broth.

Lymphocyte Cultivation

100 µl of a 5×10^6 cells/ml lymphocyte suspension was added per well of a 96-well tissue culture plate. 100 µl of APFB solution, with concentrations ranging from 5 µg/ml to 640 µg/ml, was then added. Eight replicates were carried out per concentration. Either ConA or LPS from *E. coli* was added after the cell suspension was cultivated for 12 h. The T cell mitogen ConA was added at a final concentration of 10 µg/ml. The B cell mitogen LPS derived from *E. coli* was added at a final concentration of 50 µg/ml. Meanwhile, RPMI 1640 broth alone was used as a negative control. The cultivation plate was then incubated at 37 °C and 5% CO₂ for 72 h.

Cell Proliferation Test by MTT Method

20 µl of MTT solution was added per well 4 h prior to the end of the 72 h cultivation period and returned to the incubator. After 72 h stimulation the cultivation plate was centrifuged for 5 min at 1200 g and the supernatant discarded. Excess media was absorbed using filter paper. 100 µl of acidified isopropanol was added per well and the plate incubated at room temperature for 20 min. The mixture was mixed gently and evenly, and the absorbance determined at 570 nm.

Statistical Analysis

All statistical analysis was performed using SPSS for windows 18.8 statistical software. The data were expressed as the mean ± standard deviation ($\bar{X} \pm s$). Analysis of variance (ANOVA) was used for data comparison among groups, A P-value less than 0.05 ($P < 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Separation and Purification of Acidic Polysaccharides

The crude *Boletus edulis* polysaccharide CPB was separated in the DEAE-52 cellulose column. The neutral polysaccharide fraction (NPF) eluted with deionized water and the acidic polysaccharide fraction eluted with 0.1M NaCl (Figure 1). The acidic polysaccharide fraction (APFB) was purified by Sephadex G-100 gel column chromatography, the peak collected, and the acidic polysaccharide fraction then dialyzed and lyophilized to obtain pure acidic polysaccharide APFB (Figure 2). Pure APFB was separated by ion exchange chromatography.

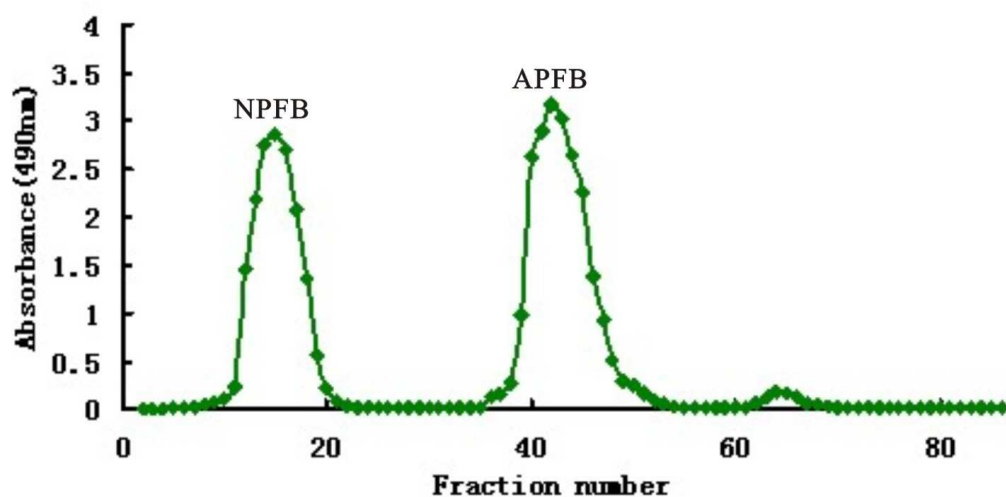


Figure 1. Chromatography of eluted crude polysaccharide (CPB) on DEAE-52 (NPFB eluted with distilled water; APFB eluted with 0.1 M NaCl)

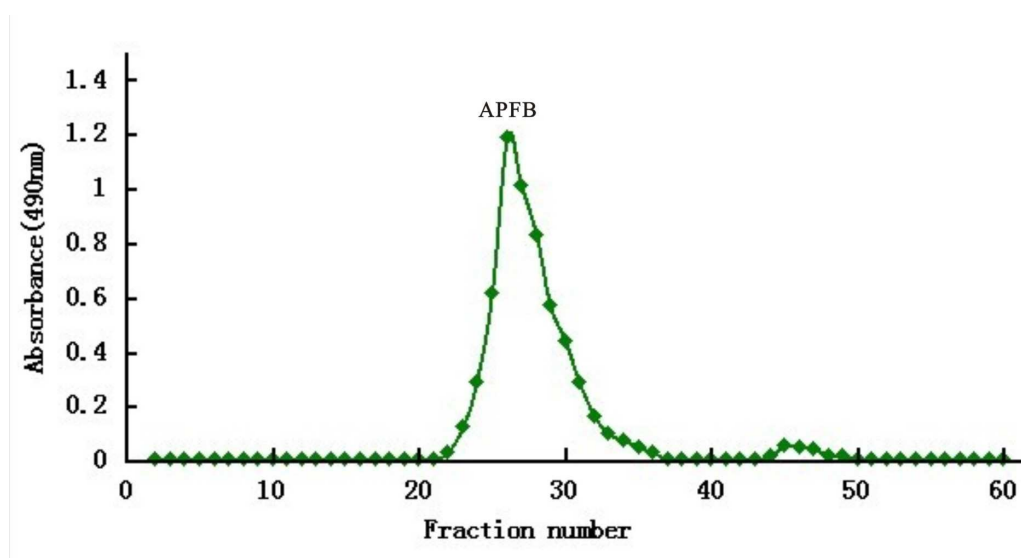


Figure 2. Chromatography of eluted acidic polysaccharide (APFB) on Sephadex G-100

APFB UV Spectral Analysis Results

No absorption peaks appeared at 260 nm or 280 nm, indicating that the APFB solution does not contain nucleic acids and proteins(Figure 3).

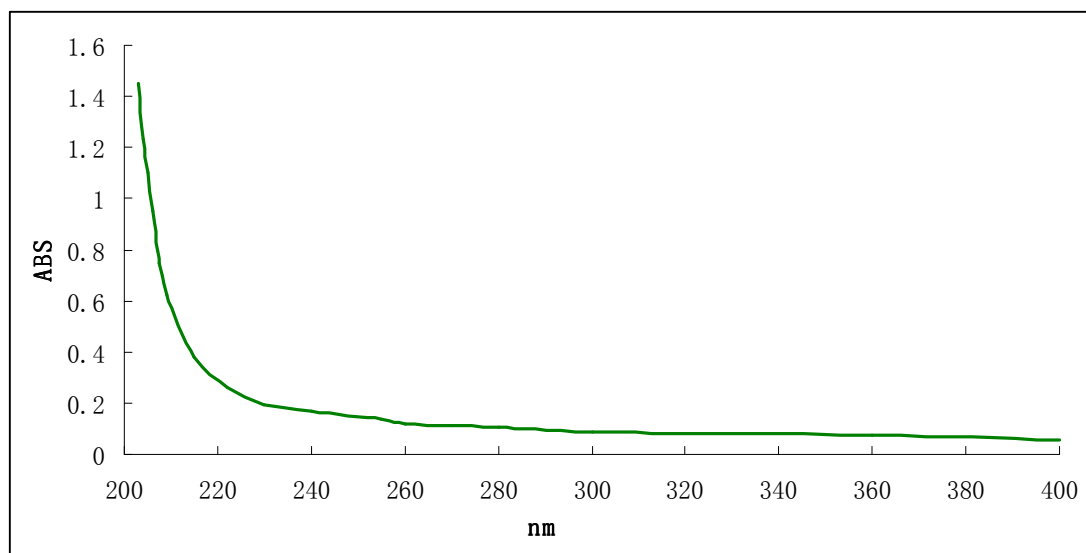


Figure 3. UV-Spectral analysis of APFB fraction

Determination of APFB Purity and Molecular Weight

A single symmetrical chromatographic peak was observed, demonstrating the homogeneous nature of the sample (Figure 4). The coordinate curve was mapped with separation retention time (RT) of different molecular weight standard dextran and corresponding molecular weight log value (LogMw). The regression equation was $\text{LogMolWt} = 9.14e - 3.69e^{-1}T$, $R = 0.999134$. APFB weight-average molar mass measured by GPC software as $M_w = 39668$, and number average molecular weight $M_n = 27625$.

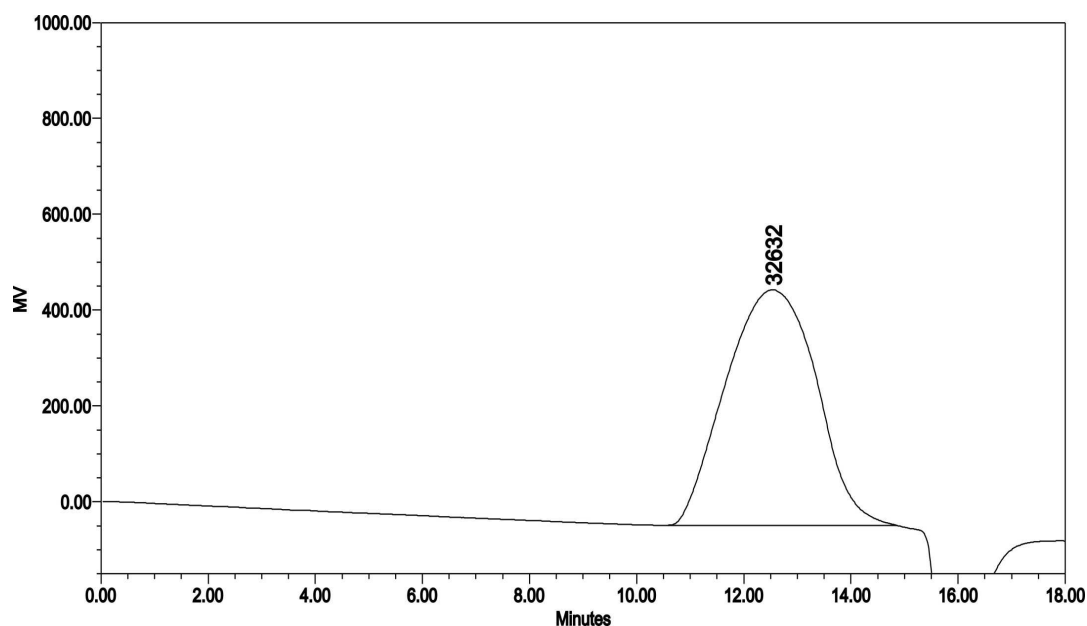


Figure 4. HPGPC chromatogram of APFB

APFB Monosaccharide Composition

The comparison with standard monosaccharide sample chromatograms by HPLC monosaccharide composition analysis showed that APFB is composed of 8 monosaccharides: mannose, xylose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose and fucose (Table 1). Galacturonic acid was the greatest fraction (32.1%) followed by glucose and galactose. The content of fucose was the lowest (1.1%), thereby demonstrating that APFB is a typical acidic heteropolysaccharide.

Table 1 HPLC Analysis APB Monosaccharide Composition

Monosaccharide	Mannose	Xylose	Rhamnose	Glucuronic ac	Galacturonic aci	Glucose	Galactose	Fucose
Retention time (min)	14.02	15.11	17.82	20.34	23.98.	30.23	33.01	34.10
Mole percentage (%)	11.8	4.2	1.5	10.6	32.1	21.4	17.3	1.1

Influence of APFB on Mouse Lymphocyte Proliferation

Mouse splenocytes were stimulated with either ConA or LPS for 72 h, and compared to the negative control of RPMI 1640 alone. Spleen T and B cells were activated and proliferated under these positive control conditions ($P < 0.05$) (Table 2).

Concentrations in the range 5 and 640 $\mu\text{g/ml}$ APFB promoted the proliferation of mouse spleen T cells as the absorption value was significantly higher than the ConA alone group ($P < 0.05$). A dose dependent effect of APFB on ConA stimulated T cell proliferation was evident between 5 $\mu\text{g/ml}$ and 160 $\mu\text{g/ml}$. When the concentration was equal to or greater than 320 $\mu\text{g/ml}$, the ability of APFB to promote T lymphocyte proliferation decreased.

APFB significantly increased the ability of LPS to promote proliferation of spleen derived B cells from 10 $\mu\text{g/ml}$ ($P < 0.05$). APFB dose-dependently increased proliferation at concentrations between 10 $\mu\text{g/ml}$ and 640 $\mu\text{g/ml}$. APFB had a maximal effect at a concentration of 320 $\mu\text{g/ml}$, with the proliferation promoting effect enhanced with the increase of concentration. However, when the concentration reached 640 $\mu\text{g/ml}$, the ability of APFB to promote cell proliferation was weak, although it was still higher than that of LPS control group ($P < 0.05$).

Table 2 Influence of APFB on Mouse Lymphocyte Proliferation (OD value, $\bar{X} \pm s$)

Group	APFB ($\mu\text{g/ml}$)	T Cell (570 nm)	B Cell (570 nm)
Blank control (1640)		0.067 \pm 0.006	0.104 \pm 0.013
ConA control		0.280 \pm 0.041 [#]	--
LPS control		--	0.402 \pm 0.029 [#]
ConA/LPS+APFB	5	0.362 \pm 0.017 [★]	0.441 \pm 0.008
ConA/LPS+APFB	10	0.373 \pm 0.025 [★]	0.498 \pm 0.031 [★]
ConA/LPS+APFB	20	0.379 \pm 0.020 [★]	0.516 \pm 0.022 [★]
ConA/LPS+APFB	40	0.391 \pm 0.044 [★]	0.543 \pm 0.024 [★]
ConA/LPS+APFB	80	0.428 \pm 0.031 [★]	0.566 \pm 0.009 [★]
ConA/LPS+APFB	160	0.430 \pm 0.019 [★]	0.579 \pm 0.026 [★]
ConA/LPS+APFB	320	0.411 \pm 0.023 [★]	0.601 \pm 0.040 [★]
ConA/LPS+APFB	640	0.366 \pm 0.038 [★]	0.532 \pm 0.042 [★]

[#]: Control comparison with 1640 ($P < 0.05$); [★]: Control comparison with T cell (ConA) or B cell (LPS) ($P < 0.05$)

CONCLUSION

Our method of separating, purifying and analyzing acidic polysaccharide from *Boletus edulis* is fast and stable with a lower sampling amount, which makes it suitable for the determination of trace polysaccharide samples.

The proliferative ability of spleen lymphocytes is an important measure to evaluate the body's immune function [20]. Our results suggest that APFB can promote humoral immune and cellular immune functions. We speculate that *Boletus edulis*-extracted acidic polysaccharides have immune regulation functions. Therefore APFB may be a kind of active immunomodulator. However, whether the anti-tumor mechanism [5] of *Boletus edulis* polysaccharide [21] and plant acidic polysaccharides is related to the promotion of lymphocyte proliferation and differentiation needs to be investigated.

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