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Structural characterization of an immunoregulatory polysaccharide from the fruiting bodies of *Lepista sordida*

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ABSTRACT

A water-soluble polysaccharide, named as LSP, was extracted and fractioned from the fruiting bodies of *Lepista sordida* by DEAE-cellulose anion exchange and Sepharose CL-6B column chromatography. Its molecular weight (Mw) was estimated to be about 4×10^4 Da by using high-performance gel permeation chromatography (HPGPC). According to Fourier transform infrared (FT-IR) spectroscopy, partial acid hydrolysis, periodate oxidation and Smith degradation, methylation and GC–MS analysis, the results indicated LSP had a backbone consisting of (1 → 6)-linked-α-D-glucopyranosyl and (1 → 2,6)-linked-α-D-glucopyranosyl residues, which was terminated with terminal (1 →)-α-D-galactopyranosyl residue at the O-3 position of (1 → 2,6)-linked-α-D-glucopyranosyl residue along the main chain in the ratio of 2:1.1:0.9. Preliminary tests in vitro showed LSP had potent activation effects on murine macrophages, supported by the fact that the elevated NO production and TNF-α secretion, and its branches played a crucial role for macrophages activation.

1. Introduction

Nowadays polysaccharides from various sources (e.g. plants, fungi and lichens) have attracted more attention in recent years due to the potential biological functions, such as antioxidant, immunostimulatory, anti-ulcer, hepatoprotective, antitumor effects and so on (Li, Chen, Wang, Tian, & Zhang, 2009; Qiao et al., 2009; Sun, 2011). Especially the polysaccharides and polysaccharide–protein complexes isolated from mushrooms have been used as a source of therapeutic agents (Ooi & Liu, 2000). Several investigators have isolated and purified immunomodulatory polysaccharides from mushrooms as a biological response modifier (BRM) i.e. they cause no harm and place no additional stress on the body, but help the body to adopt to environmental and biological stress (Mizuno et al., 2000). Recently, Lentinan, Schizophyllan, Polysaccharide-K (PSK), and Polysaccharide-P (PSP) have been reported to have effectively inhibiting abilities on the growth of various transplantable tumors in experimental animals and increase the survival rate (Liu, Li, Kong, Lin, & Gao, 1998). Furthermore, several species of fungi are used as traditional medicines in treatment of different human diseases such as hepatitis, hypertension, hypercholesterolaemia, and gastric cancer (Park, Kim, Hwang, & Yun, 2001). Mushrooms comprise a vast and yet largely untapped source of modern medicine and they represent an unlimited source of polysaccharides with various biological properties. Therefore, discovery and evaluation of new polysaccharides from the various medicinal mushrooms as new safe compounds for functional foods or medicine has become a hot research spot.

Lepista sordida (Schum.: Fr.) Sing is a kind of delicious edible mushroom distributed mainly in the Province of Heilongjiang, which is a Lentinus fungus belonging to the Basidiomycetes. Two diterpenes (lepistal and lepistol) with the activity to induce differentiation in human leukaemia cells were previously isolated from the culture filtrate of this fungus (Mazur, Becker, Anke, & Sterner, 1996). Moreover three new 3, 6-dioxygenated diketopiperazines, lepistamides A–C, along with a known compound, diatretol, were isolated from the mycelial solid cultures of the basidiomycete *L. sordida* (Chen, Wu, Ti, Wei, & Li, 2011). However, to date, there is no investigation carried out on the isolation and structural elucidation of polysaccharides the fruiting bodies of *L. sordida*.

To the best of our knowledge, structure and functions are intimately related. Therefore as a prerequisite to better understand its biological activities and underlying action mechanism, the aim of this work was mainly to report on the extraction and purification of a water-soluble polysaccharide from the fruiting bodies of *L. sordida*, and elucidate its structural characterization by a combination of chemical and instrumental analyses. In addition, the macrophages activation induced by LSP and the

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backbone of LSP (the weak acid-hydrolyzed product of LSP) was estimated via determining the NO production and TNF-α secretion.

2. Experimental

2.1. Materials

The fruiting bodies of *L. sordida* were purchased from local market in Chengdu. Sepharose CL-6B and DEAE-cellulose was purchased from Amersham Pharmacia Co. (Sweden). Medium RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) and endotoxin-free fetal bovine serum (FBS) were purchased from Gibco Invitrogen Co. N-[1-naphthyl]-ethylenediamine, trifluoroacetic acid (TFA), D-glucose, lipopolysaccharide (LPS) and T-series dextran was purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

2.2. General methods

UV–vis absorption spectra were recorded with a UV-Vis spectrophotometer (Model SP-752, China). FT-IR was recorded on SPECORD in a range of 400–4000 cm^{-1} (KBr pellets). Gas chromatography (GC) was performed on a Varian 3400 (Hewlett-Packard Component, USA) equipped with DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min, and increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. The injector and detector heater temperature were 250 and 300 °C, respectively. The rate of N_2 carrier gas was 1.2 ml/min. Gas chromatography–mass spectrometry (GC–MS) was done on a Shimadzu QP-2010 instrument (Shimadzu, Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm), and at temperatures programmed from 120 °C (maintained for 2 min) to 260 °C (kept for 40 min) at a rate of 15 °C/min. The total carbohydrate content was determined by the phenol–sulfuric acid method using D-glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Micro-Kjeldahl method (Wang, Qin, Gao, & Yan, 1999). Uronic acid content was determined according to an m-hydroxydiphenyl colorimetric method by using D-galacturonic acid as the standard (Filisetti-Cozzi & Carpita, 1991). Dialysis was carried out using tubing with a Mw cut-off of 500 Da (for globular proteins).

2.3. Isolation and purification of polysaccharide

The fruiting bodies of *L. sordida* (500 g) were defatted under reflux with absolute alcohol (5000 ml) at 75 °C for 3 times and 2 h for each time. After the combined extraction solution was filtered, the residues were dried and extracted with distilled water at 80 °C for 3 times and 2 h for each time. The whole extract solution was filtered and centrifuged before concentration, and then submitted to precipitation with three volumes of ethanol at 4 °C overnight. The precipitate dissolved in H_2O, collected by centrifugation, was deproteinated by freeze thawing, proteinase digestion and the Sevag method (Staub, 1965), and exhaustive dialyzed with water for 48 h. Finally the concentrated supernatant was lyophilized to give crude *L. sordida* polysaccharides, coded as CLSP.

The CLSP was dissolved in distilled water and filtered. After the filtering solution was loaded onto DEAE-cellulose anion-exchange chromatography column (3 cm × 30 cm), the column was eluted with continuous gradient concentrations of NaCl aqueous solution (0.15 → 2 M, PH = 6–7) stepwise at 4 ml/min, and each tube fraction was collected by the automated fraction collector. The water eluted fraction was collected and dialyzed. Then the sample was further purified on a Sepharose CL-6B column (2.6 cm × 100 cm) with 0.15 M NaCl at a flow rate of 1 ml/min to yield one main fraction (LSP). The LSP was collected, dialyzed and lyophilized to give white purified polysaccharide fraction. Total carbohydrate and protein content of each tube was measured at 490 nm by Dubois's method and Micro-Kjeldahl method, respectively.

2.4. Assays for structural analysis

2.4.1. Partial hydrolysis with acid

Polysaccharide sample was hydrolyzed with 0.05 M trifluoroacetic acid, kept at 95 °C for 16 h, and centrifuged to yield one precipitation (the backbone of LSP), named as LSPA. After TFA was removed by evaporation, the remains were dialyzed with distilled water for 48 h, and then diluted the solution in the sack with ethanol. After hydrolyzation, the precipitate and supernatant in the sack and the fraction out of sack were dried, and then GC analysis was carried out (Sun, Liang, Zhang, Tong, & Liu, 2009). LSPA was subjected to the following assay with LSP for macrophages activation by polysaccharides.

2.4.2. Periodate oxidation and Smith degradation

The periodate oxidation was performed according to the procedure described by Sun et al. (2009). The sample (25 mg) was dissolved in 0.015 M $NaIO_4$ (25 mL), and the solution was kept at in the dark at the room temperature. 0.1 ml reaction solution was withdrawn at 3–6 h intervals, diluted to 25 ml with distilled water and determined in a spectrophometer at 223 nm (Alfred, Leigh, & Giuseppe, 2001). After the oxidation was finished (70 h), the excessive $NaIO_4$ was terminated with glycol (2 mL). The solution of periodate product (2 ml) was sampled to calculate the yield of formic acid by 0.01 M sodium hydroxide, and the rest was extensively dialyzed against tap water and distilled water for 24 h, respectively. The solution was concentrated and reduced with $NaBH_4$ (60 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and was concentrated to a volume (10 ml). One-third of solution described above was freeze dried and analyzed with GC. Others were added to the same volume of 1 M sulfuric acid, maintained for 40 h at 25 °C, neutralized to pH 6.0 with barium carbonate, and filtered. The filtrate was dialyzed as foresaid, and the content out of the sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and precipitate were also dried out for the GC analysis.

2.4.3. Monosaccharide composition

GC was used for identification and quantification of the composition of polysaccharide samples were hydrolyzed and acetylated according to the method by Lehrfeld (1985). Firstly, the samples (10 mg) were hydrolyzed with 2 M TFA (2 ml) at 120 °C for 2 h, and the excess acid was completely removed by co-distillation with ethanol. Then the hydrolyzed product was reduced with KBH_4 (30 mg), followed by neutralization with dilute acetic acid and evaporated at 45 °C after adding 1 mg myo-inositol and 0.1 M Na_2CO_3 (1 ml) at 30 °C with stirring for 45 min. After desalted by cation exchange resin, filtered by quantitative filter paper and neutralized by evaporating with methanol, the reaction solution was mixed with 1 mL n-propylamine and anhydrous pyridine for 30 min at 55 °C. Finally dried sample was mixed into 0.5 mL anhydrous pyridine and acetic anhydride for 1 h at 90 °C (Jones & Albersheim, 1972; Oades, 1967). The acetylated products were analyzed by GC as previously mentioned (Sun et al., 2008), and identified and estimated with myo-inositol as the internal standard.

2.4.4. Homogeneity and M_w determination of polysaccharides

The averaged molecular weight was determined by HPGPC (Sun et al., 2008), which was performed on a SHIMADZU system with a TSK-G3000PWXL column (7.8 mm × 30.0 cm) and a SHIMADZU RID-10A detector. 0.7% Na_2SO_4 was chosen as eluent buffer and the flow rate was 0.7 ml/min at 40 °C with 1.6 mpa. The averaged molecular weight was estimated according to a calibration curve made from a set of Dextran standards (T 130, 80, 50, 20, 10).

2.4.5. Methylation analysis

The polysaccharides were methylated three times by reference to the method described by Needs and Selvendran (1993), and the resulting methylated products were depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 100 °C for 2 h. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The partially methylated residues were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were identified by their fragment ions in GC–MS and by relative retention times on GC, and the molar ratios were estimated from the peak areas and response factors.

2.5. The effect of polysaccharide on the NO production and TNF-α secretion in vitro

Murine macrophage J774A.1 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated, endotoxin free FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultivated in sterile tissue culture flasks and gently detached by scraping.

J774A.1 cells were plated at a density of 1.5 × 105 cells/well in a final volume of 200 μl in 96-well flat-bottom tissue culture plates and incubated in medium only or in medium containing various concentrations (50 μg/ml and 100 μg/ml) of polysaccharide fractions (LSP and LSPA) or *Escherichia coli* LPS (1 μg/ml) as a positive control for 24 h. Production of nitric oxide (NO) was estimated by measuring nitrite levels in cell supernatant with 100 μl of Griess reagent (1% sulfanilamide in 2.5% phosphoric acid, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples. The nitrite concentration was determined at 540 nm using $NaNO_2$ as a standard (Da Silva et al., 2009).

The ability of LSP and LSPA to induce production of TNF-α in peritoneal macrophages was determined by dissolving the polysaccharide in the culture medium at the concentration of 50 and 100 μg/ml. After pre-incubation peritoneal macrophages in 48-well culture plates for 24 h, supernatants were harvested and the concentration of TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions. LPS (1 μg/ml) was designated as positive control.

2.6. Endotoxin determination

Endotoxin was measured by Limulus amebocyte lysate assay (Pyrochrome®, Associates of Cape Cod Inc., Woods Hole, USA) according to the manufacturer's instruction.

3. Results and discussion

3.1. Isolation, purification and chemicophysical properties of LSP

After the crude polysaccharides chromatographed on the DEAE-cellulose anion exchange and Sepharose CL-6B column in sequence, a water-soluble polysaccharide was purified from the fruiting bodies of *L. sordida*, named as LSP. LSP appeared as a white powder and showed a single and symmetrically sharp peak on HPGPC (Fig. 1), indicating it was a homogeneous polysaccharide. Its molecular weight was estimated to be 4×10^4 Da by HPGPC according to the calibration curve with standard dextran and glucose. It had a negative response to the Micro-Kjeldahl test and no absorption at 280 or 260 nm in the UV spectrum, revealing the absence of protein and nucleic acid. Total carbohydrate content was determined to be 93% determined by phenol–sulfuric acid method. GC analysis showed LSP was composed of D-glucose and D-galactose in molar ratios of 3:1 (data not shown). The results indicated that the glucose was the predominant monosaccharide.

Table 1
GC analysis for fractions from partial acid hydrolysis.

Fractions	Molar ratios			
	Gly	Ery	Glc	Gal
LSPA (precipitation after hydrolysis)	–	–	2.87	–
LSPB (precipitation in the dialysis sack)	–	–	0.23	–
LSPC (supernatant in the dialysis sack)	–	–	–	0.1
LSPD (fraction out of dialysis sack)	–	–	–	1.0

Gly, glycerol; Glc, glucose; Gal, galactose.

The distinctive polysaccharide absorption of LSP in IR bands appeared at 1000–1150 cm^{-1}, 1400–1550 cm^{-1}, 2800–2900 cm^{-1}, and 3100–3500 cm^{-1}. The strong band in the region of at 3412.3 cm^{-1} was attributed to the hydroxyl stretching vibration of the polysaccharide, and the bands at 2875.1 cm^{-1} and 1634.3 cm^{-1} were due to the C–H stretching vibration absorption and associated water, respectively. Characteristically, the bands at 1000–1100 cm^{-1} suggested the presence of pyranose form of the glucosyl residue in LSP. The absorption band at 846.3 cm^{-1} confirmed the existence of sugar α-linked residues in LSP (Fig. 2), which was in good agreement with the results of GC/MS analysis.

3.2. Structural analysis of LSP

3.2.1. The results of partial hydrolysis with acid

All the products after partial acid hydrolysis of LSP, including LSPA, LSPB, LSPC and LSPD were subjected to GC analysis. From Table 1 we can conclude that Glc in LSPA and LSPB comprised the backbone component of LSP, and Gal in LSPC and LSPD existed as the branched or terminal residues of LSP.

3.2.2. The results of periodate oxidation and Smith degradation

Result from the periodate oxidation experiment showed that more amount of $NaIO_4$ was consumed, when LSP was oxidized. 1 mol of sugar residue consumed $NaIO_4$ (0.631 mol) and produced 0.265 mol of formic acid. The consumption of $NaIO_4$ was about two times more than the amount of formic acid that was produced after 70 h of periodate treatment, indicating that LSP not only contained linkages which can produce formic acid such as 1 → and 1 → 6, but also contain some amount of linkage type which only can consume $NaIO_4$ such as 1 → 2, 1 → 4, 1 → 2, 6, and 1 → 4, 6. The periodate-oxidized products were fully hydrolyzed and examined by GC analysis (Table 2). The predominant presence of glycerol (Gly)

Table 2
GC results from fractions of Smith-degradation of LSP.

Fractions	Molar ratios		
	Gly	Glc	Gal
Full acid hydrolysis	4.39	–	–
Out of sack	1.11	–	–
Supernatant in sack	–	–	–
Precipitation in sack	–	–	–

Gly, glycerol; Glc, glucose; Gal, galactose.

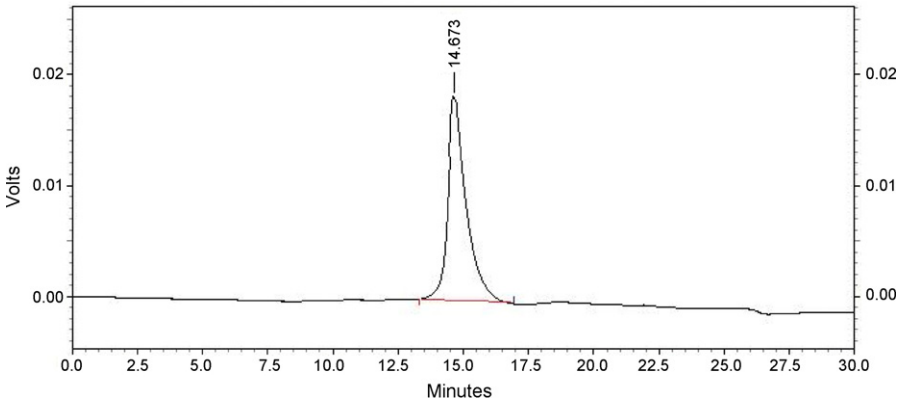

Fig. 1. Profile of LSP in HPGPC.

revealed that all sugar residues of Glc and Gal were (1 →)-linked, (1 → 2)-linked, (1 → 6)-linked, or (1 → 2, 6)-linked linkages that can be oxidized to produce only Gly. Likewise no Glc and Gal can be detected by GC proved that all residues in LSP can be oxidized by periodate. In addition, the fraction out of sack also showed Gly peak in GC profile. However there was no precipitation in the sack and not any peak was found in GC. Hence combined with the conclusion of partial acid hydrolysis of LSP, we can concluded that the terminal residues of LSP should be (1 →)-linked Gal and the Glc residues in backbone of LSP should existed in the following form, such as (1 →)-linked, (1 → 2)-linked, (1 → 6)-linked, or (1 → 2, 6)-linked linkage.

3.2.3. The results of methylation analysis

For the purpose of further determining the structural features of LSP, the GC–MS as an effective and conventional method was introduced to the experiment. According to ion fragment peak and peak areas from Table 3, three sugar residues can be identified to (1 → 6)-linked-α-D-glucopyranosyl (Residue-A), (1 → 2, 6)-linked-α-D-galactopyranosyl residues (Residue-B) and (1 →)-α-D-galactopyranosyl (Residue-C) groups in the ratio of 2:1.1:0.9. In the light of the results of partial hydrolysis with acid, periodate oxidation and Smith degradation and GC–MS, it was possible to conclude that a repeating unit of LSP contained the backbone chains with Residue-A and B, and Residue-C as a terminal residue substituted in O-2 position of Residue-B.

3.3. Assay for macrophages activation by polysaccharides

3.3.1. The effect of polysaccharide on the macrophage NO production in vitro

The effect of LSP and LSPA on the NO production in murine macrophage J774A.1 cells was shown in Fig. 3, a small amount of NO was produced when macrophages were exposed to medium alone or LSPA at the concentration of 50 and 100 μg/ml, however incubation of these cells with LSP at the same concentration induced a significant increase of NO release ($P < 0.05$ or $P < 0.01$) in a dose-dependent manner compared with negative control. Moreover the level of NO production at 100 μg/ml was comparable to that elicited by LPS at 1 μg/ml.

Table 3
GC–MS analysis of methylated LSP.

Peak no.	Methylated sugar	Molar ratio	Linkage type
1 (Residue-A)	2,3,4-Me_3-Glc*p*	20	→ 6)-α-Glc*p*-(1 →
2 (Residue-B)	3,4-Me_2-Glc*p*	11	→ 2,6)-α-Glc*p*-(1 →
3 (Residue-C)	2,3,4,6-Me_4-Gal*p*	9	α-Gal*p*-(1 →

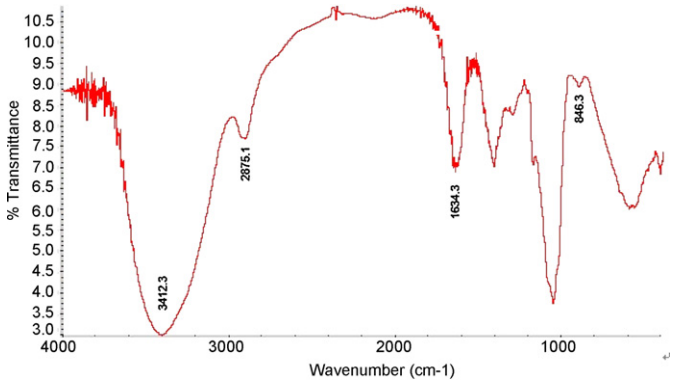

Fig. 2. FT-IR spectra of the polysaccharide of LSP.

3.3.2. The effect of polysaccharide on the macrophage TNF-α production in vitro

To examine whether LSP and LSPA activated macrophages to produce cytokines, the amounts of TNF-α were measured by ELISA. As seen from Fig. 4, on the one hand the visible augment of TNF-α releasing ($P < 0.01$) was triggered by LSP at the high dose of 100 μg/ml as compared to the control, which was up to that in LPS-treated group, on the other hand, there was no any increase in TNF-α production in macrophages treated by LSPA.

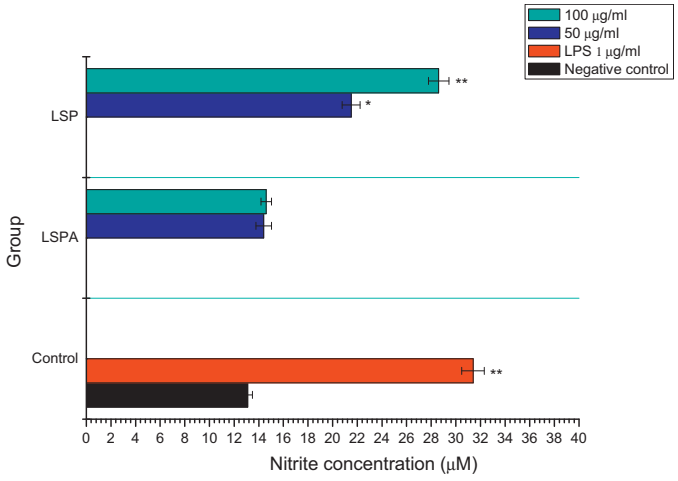

Fig. 3. Effect of LSP and LSPA on the macrophage NO production in vitro. Values were presented as mean value ($n = 3$).

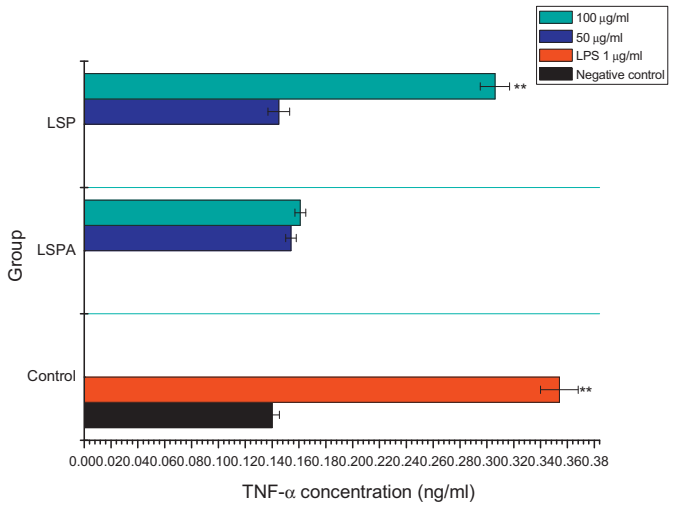

Fig. 4. Effect of LSP and LSPA on the macrophage TNF-α production in vitro. Values were presented as mean value ($n = 3$).

3.3.3. Endotoxin content

To the best of our knowledge, LPS could exhibit various immunological activities, including immune cell activation, thus LPS contamination may lead to false positive result of biological tests. In order to overcome this problem, we determined the endotoxin content and the result showed all samples contain no endotoxin, which proved that all polysaccharide samples rather than the endotoxin were contributed to the macrophages activation potency.

4. Conclusion

In this report, we obtained one purified polysaccharide from the fruiting bodies of *L. sordida* by DEAE-cellulose anion exchange and Sepharose CL-6B column chromatography, and identified its structural characteristic by combination of chemical and instrument analysis as following: the backbone consisted of the repeating disaccharide [→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→2,6)-α-D-Glc*p*-(1→], which was terminated by [→1)-α-D-Gal*p*] attached to the backbone through O-2 of Gal residues in the ratio of 2:1.1:0.9. Macrophages are the major source of TNF-α and NO, and participate in fighting against infectious agents and tumor cells. Moreover macrophages could be activated to become cytotoxic by a set of cytokine signals. NO is known to play a key role during the course of infections (MacMicking, Xie, & Nathan, 1997) and TNF-α is believed to be one of the strongest antitumor factors cell until now. One of the most prominent characteristics of TNF-α is its ability to cause apoptosis of tumor-associated endothelial cells, resulting in tumor necrosis (Lejeune, Lienard, Matter, & Ruegg, 2006). TNF-α also plays a pivotal role in host defense and induce the expression of a number of other immunoregulatory and inflammatory mediators (Baugh & Bucala, 2001). In the present study we observed that LSP could significantly increase NO and NF-α release from macrophages, whereas LSPA did not work to activate macrophages. Therefore, we can infer that the branched sugar residue is crucial to the activation for macrophages during the process of immunoregulation, which could contribute to the more contact area of polysaccharide to the receptor in the surface of macrophages.

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