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Structure and Immuno-Stimulating Activities of a New Heteropolysaccharide from *Lentinula edodes*

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ABSTRACT: In this study, a new heteropolysaccharide, here called L2, was separated from the fruit body of *Lentinula edodes*. Chemical and physical analyses indicated that L2 has a molecular weight of 26 KDa and consists of glucose (87.5%), galactose (9.6%), and arabinose (2.8%), but it does not possess a triple-helical conformation. Stimulation of the production of nitric oxide (NO), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) in RAW 264.7 cells showed that L2 has significant immune activities involving toll-like receptor 2 (TLR2). Further studies demonstrated that the activities of L2 exhibited high stability in wide range of pH from 4.0 to 10.0, when the thermal processing temperature was below 121 °C. Our findings revealed that a new heteropolysaccharide without triple-helical conformation from *Lentinula edodes* shows immuno-stimulating activities involving TLR2 at modest pH and thermal processing conditions, which enable it to act as an active component in foods.

KEYWORDS: *mushroom, Lentinula edodes, heteropolysaccharide, structure, immuno-stimulating activity, toll-like receptor*

INTRODUCTION

Mushrooms have been used as medicine and food for thousands of years in East Asia. During the past few decades, many active substances from mushrooms have been reported to have antitumor and immuno-modulating activities. These substances include proteins, polysaccharides, and protein−polysaccharide complexes.[1−3] Because of the absence of side effects, polysaccharides are considered biological response modifiers for their antitumor and immuno-modulating activities. These abilities were found to be associated with solubility, monosaccharide composition, molecular weight, degree of branching, and tertiary conformation.[4]

Lentinula edodes is cultivated largely in China and Japan. It is the second most popular mushroom in the world.[5] Since the 1970s, several therapeutic polysaccharides from *Lentinula edodes*, lentinan, LAP, and KS-2, which have been shown to have pharmacological properties, have been the subjects of formal studies and have seen use in medical practice.[6,7] Lentinan is a typical β-glucan with a 1→3 linked main chain. Its chemical structure and antitumor and immuno-modulating activities are fully understood.[8] Although β-glucan from the fruit body of *Lentinula edodes* has been deeply investigated, heteropolysaccharides have only seldom been explored because of their complicated structures.[8] To date, several heteropolysaccharides have been isolated from the fruit body of *Lentinula edodes* with different extraction methods.[9−12] However, the details of their chemical structure and immuno-modulating activities are rarely investigated.

Macrophages are one of the most important immune cells. They play pivotal roles in the defense against microbial infection and tumorigenesis. Macrophages are usually the preferred tools for the examination of the immuno-modulating properties of polysaccharides.[13,14] NO is produced through the activation of inducible oxide synthase (iNOS), which is regulated by nuclear factor-kappa B (NF-κB) in murine macrophages. Upon activation, macrophages first synthesize and secrete proinflammatory cytokines including TNF-α and IL-6.[15] For this reason, NO, TNF-α, and IL-6 are usually used as immune response parameters in macrophage stimulation experiments.[13,14]

In the present study, a new heteropolysaccharide L2 was isolated from the fruit body of *Lentinula edodes*. We determined its chemical structure and conformation and examined its immuno-stimulating activities and immune receptors using the mouse macrophage cell line RAW264.7. The effects of different pH values and thermal treatments on the activity of L2 were also investigated.

MATERIALS AND METHODS

Extraction and Purification of Polysaccharide from the Fruit Body of *Lentinula edodes*. The dried fruit body of *Lentinula edodes* was collected from Suizhou, Hubei Province, China. It was identified as *Lentinula edodes* (Berk.) Pegler by Dr. Hongwei Liu from the Institute of Microbiology of the Chinese Academy of Sciences. The fruit body of *Lentinula edodes* was extracted with boiling water over the course of 2 h at a ratio 20:1 (w/w). After two rounds of extraction, the supernatants were combined and concentrated under 80 °C. The resulting solution was adjusted using 95% ethanol to a concentration of 75% ethanol and kept overnight at room temperature. It was then separated on a centrifuge. The precipitates were dried under vacuum. Ten grams of dried precipitate was dissolved in 100 mL of distilled water, followed by deproteination using the Sevag method.[16] This process was repeated five times. Three volumes of 95% ethanol were added to the resulting solution, which was centrifuged and redissolved in distilled water. This procedure was repeated three times. The resulting solution was then freeze-dried at −40 °C to obtain crude polysaccharides.

DEAE (diethylaminoethyl)-52 cellulose column chromatography was used for further separation. The crude polysaccharides (160 mg) were dissolved in 8 mL of distilled water, applied to a DEAE-52 cellulose anion exchange column (60 cm × 2.5 cm), and eluted with

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distilled water, 0.05 mol/L NaCl solution, 0.1 mol/L NaCl solution, and 0.2 mol/L NaCl solution, respectively. The eluent was collected using an automatic collector and detected by the phenol−sulfuric acid method. The elution curve was drawn by the tube number and absorbance (490 nm). Eluents with the same peak were combined, concentrated at 50 °C, and dialyzed against distilled water for 48 h. Four fractions, CL1, CL2, CL3, and CL4, were obtained by freeze-drying. The main fraction, CL2, showed the most pronounced ability to stimulate NO production in RAW 264.7 cells (data not shown). For this reason, CL2 was selected for further purification and study. Twenty milligrams of CL2 was dissolved in 2 mL of distilled water and loaded onto a Sephadex G-200 column. The sample was eluted with 0.05 mol/L NaCl solution at a ratio of 1.5 mL/10 min. The eluent was collected with automatic collector and examined using the phenol−sulfuric acid method. The elution curve was drawn using the tube number and absorbance (490 nm). The elution peaks were evaluated, and the L2 fraction was collected after gel filtration, dialysis, and freeze-drying. The L2 powder was white in color. Lipopolysaccharide (LPS) contamination was evaluated using Pyrotell G2250 kits (Associates of Cape Cod, Inc., East Falmouth, MA), and no contamination was detected. The carbohydrate content of L2 was 97% as indicated by phenol sulfuric acid analysis with glucose as a standard. L2 was free of nucleic acid and protein as indicated by ultraviolet scanning.

Monosaccharide Composition. The monosaccharide composition of L2 was determined as previously reported.[17] Glucose, galactose, mannose, xylose, fucose, rhamnose, and arabinose were used as the monosaccharide standards (Sigma-Aldrich Chemie, Steinbeim, Germany).

Determination of Molecular Weight. The molecular weight of L2 was measured using high-performance gel permeation chromatography (HPGPC) performed on a Waters instrument equipped with TSK-GEL G-5000 PW_{XL} column (300 mm × 7.8 mm i.d., 10 μm) and TSK-GEL G-3000PW_{XL} column (300 mm × 7.8 mm i.d., 6 μm) connected in series, eluted with 0.02 moL/L KH_2PO4 at a flow rate of 0.6 mL/min. The column temperature was kept at 35 ± 0.1 °C. A standard curve was set up using Dextran-5, Dextran-12, Dextran-25, Dextran-50, Dextran-270, Dextran-400, Dextran-410, Dextran-510, and Dextran-670 (Sigma-Aldrich Production GmbH, Buchs, Switzerland). Twenty microliters of solution was analyzed in each run.

Infrared Spectrum. The infrared profile of L2 was determined by the KBr-disk method in the range of 400−4000 cm^{-1}.[18]

Periodate Oxidation−Smith Degradation. The samples were treated using a modified version of the method described by Chi.[18] Twenty milligrams of L2 was dissolved in 12.5 mL of distilled water, and 12.5 mL of $NaIO_4$ (30 mmol/L) was then added. The solution was kept in the dark at room temperature, and 0.1 mL of aliquots were withdrawn at 6−12 h intervals, diluted to 100 mL with distilled water, and read using a spectrophotometer at 223 nm, until the optical density value became stable. Glycol (2 mL) was used to stop periodate oxidation. The solution of periodate product (2 mL) was titrated to calculate the production of formic acid by 0.01 mol/L NaOH, and the rest was extensively dialyzed against tap water and distilled water for 48 h. The residue was concentrated and reduced with $NaBH_4$ (70 mg). The solution was placed at room temperature for 24 h, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and concentrated to a volume of 10 mL followed by freeze-drying. Subsequent treatments were performed as described previously.[17] The residues (10 mg) were hydrolyzed with 4 mL of 2 M trifluorocetic acid in a sealed glass tube at 120 °C for 8 h. Acetylation was carried out with 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine for 30 min at 90 °C. Next, 0.5 mL of acetic anhydride was added with continuously heating. The acetate derivative was analyzed using an Aglient gas chromatograph 6890N system with a HP-45 column (30 m × 0.32 mm i.d., 0.25 μm) and a flame ionization detector. The temperature program was set to increase to 220 °C from 80 °C at an increment of 2 °C/min, then elevating to 250 °C at an increment of 5 °C/min and holding for 5 min at 250 °C. The detector was set at 250 °C. The injection volume was 1.0 μL. Glucose, galactose, glycol, glycerol, and erythrite were used as standards.

Determination of Tertiary Structure with Congo Red. The conformational structure of L2 was determined using the Congo red method described previously.[19]

Preparation of L2 Samples for Cell Culture. (1) L2 solutions were made in phosphate-buffered saline (PBS) at concentrations of 62.5, 125, 250, 500, and 1000 μg/mL. (2) 6 mL of L2 in PBS at a concentration of 250 μg/mL was divided into three parts and thermal treated for 30 min at 100, 121, and 137 °C, respectively. (3) L2 was dissolved in phosphate buffer at pH 4.0, 6.0, 8.0, 10.0, and physiological pH at a concentration of 250 μg/mL. All samples then were placed overnight at room temperature. (4) 250 μg/mL of L2 in phosphate buffer at pH 4.0, 6.0, 8.0, 10.0, and physiological pH were treated at 121 °C for 30 min. All solutions were sterilized using a 0.45 μm filter and then added to the well and incubated with RAW 264.7 cells.

Cell Culture and Stimulation. RAW264.7 cells were purchased from Medical College of Sun Yat-Sen University and cultured with RPMI (Roswell Park Memorial Institute) 1640 medium plus the 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. Cells in the logarithmic growth phase were adjusted to a concentration of 1 × 106 cells/mL. The cell solutions (100 μL) and sterilized PBS (100 μL) were added to each well. Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere cultivator for 24 h. The culture medium was then refreshed, and cells were incubated with L2 samples. Anti-TLR2 antibody (Abcam Inc., Cambridge, MA), anti-TLR4 antibody (Abcam Inc., Cambridge, MA), anti-CR3 antibody (eBiosience Inc., San Diego, CA), and laminarin (Sigma-Aldrich Inc., St. Louis, MO) were used to investigate the immune receptors of L2 on the RAW264.7 cells. RAW264.7 cells were treated with antimouse TLR2 (5 μg/mL), antimouse TLR4 (5 μg/mL), antimouse CR3 (5 μg/mL), and laminarin (500 μg/mL) for 1 h before stimulation with L2 and incubation for 24 h. LPS (50 μg/mL) served as the positive control. The supernatants were collected after 24 h incubation. The concentrations of TNF-α, IL-6, and NO were determined.

Measurement of Concentrations of NO, TNF-α, and IL-6. NO concentration in the culture medium supernatant of RAW 264.7 cells was detected as total nitrite using the Greiss method.[19] One hundred microliters of supernatant was mixed with 100 μL of Greiss reagents. After incubation for 10 min at room temperature, absorbance was determined at 540 nm. A standard curve was prepared using sodium nitrate (0−200 μmol/L). Concentrations of TNF-α and IL-6 were determined using a Mouse IL-6 ELISA Kit (Neobiosience Technology Co., Ltd., Shenzhen, China) and Mouse TNF-α ELISA Kit (Neobiosience Technology Co., Ltd., Shenzhen, China) according to the manufacturer instructions. All reagents used in this study were of the highest quality available in China.

Statistical Analysis. Data are presented as mean ± SD and were analyzed for statistical differences. Student's *t* tests were used for all statistical analysis between different groups. *P* values below 0.05 were considered significant.

RESULTS AND DISCUSSION

Determination of L2 Structure. Over the past several decades, more attention has been paid to the polysaccharides from mushrooms for their antitumor and immuno-modulating activities.[1,2] To date, proteoglycan, β-glucan, α-glucan, and β-heteropolysaccharide have been separated from the fruit body of *Lentinula edodes* using different methods.[6−12,20−22] Because the activity of polysaccharide relies on the structure, which includes monosaccharide composition, position of glycosidic linkages, and tertiary conformation in solution, the chemical structure and conformation of L2 were determined to understand the immunostimulating properties.[4]

Chemical Structure of L2. Using acid hydrolysis, gas chromatography (GC), and HPGPC, the polysaccharide L2 was found to consist mainly of glucose (87.5%), galactose (9.6%), and arabinose (2.8%) and to have an average molecular weight of 26 kDa (Figure 1). Infrared (IR) spectroscopy indicated absorption at 3392, 2917, and 1420 cm^{-1}, corresponding to the stretching

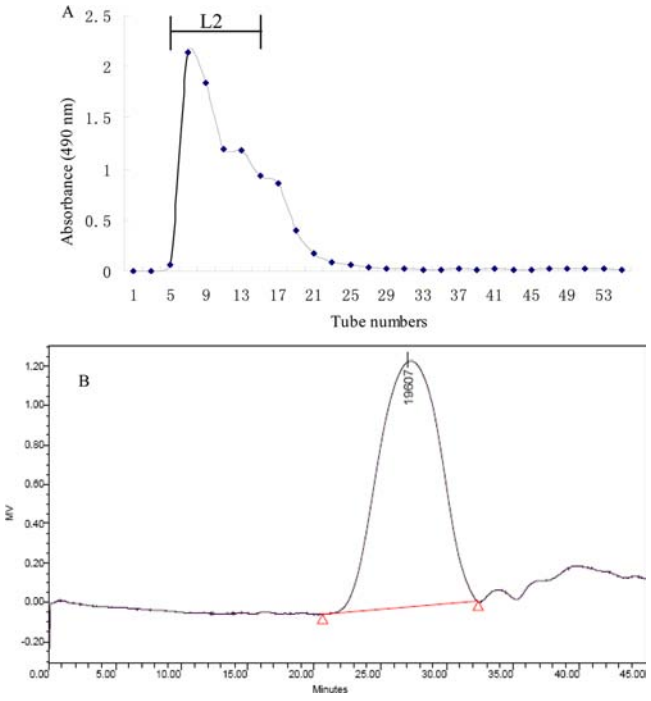

Figure 1. Chromatography of L2 by (A) Sephadex G-200 and (B) HPGPC. The weight-average molecular weight and peak molecular weight of L2 are 26 and 19 KDa, respectively.

of the O−H, C−H, and carboxyl C−O groups. There was no absorption at 1750−1700 cm^{-1}, demonstrating the absence of uronic acid (data not shown).

The position of glycosidic linkages in L2 was also examined. The results of periodate oxidation showed 1.069 mol of HIO_4 to be required to produce 0.445 mol of formic acid for 1 mol of hexose residue, indicating the 1→ or 1→6 glycosidic linkages accounted for 44.5% and 1→2 or 1→4 glycosidic linkages accounted for 17.9% of all linkages in the molecule. The periodate-oxidized and Smith-degraded products were further analyzed by GC. The presence of glucose indicated that some of the linkages were (1→3)-linked, (1→2, 3)-linked, (1→2, 4)-linked, (1→3, 4)-linked, (1→3, 6)-linked, and (1→2, 3, 4)-linked through glucose, accounting for about 37.6% of all linkages in the molecule. These linkages could not be oxidized. Neither arabinose nor galactose was detected.

The chemical analysis results demonstrated that L2 showed different monosaccharide composition and branching linkages from previous research results.[9−12] In mushrooms, polysaccharides are present as structural components of the cell walls. Shida et al.[23] proposed that the cell wall of the fruit body of *Lentinula edodes* can be divided into three layers: the outer layer, which is heteropolysaccharide and β-(1→3)-glucan with β-(1→6)-branches; the middle layer, which is mainly β-(1→6)-glucan with small numbers of β-(1→3) branches; and the inner layer, which is a complex of chitin, β-glucan, and a small amount of acid polymer. Polysaccharides isolated from the fruit body using the same method but collected from different places exhibited similar structures.[24] This showed that the solvent and temperature of extraction methods can affect the amount and structure of polysaccharides stripped from the cell walls very significantly. The differences in extraction method and purification procedure may be the cause of the structural differences between L2 and other reported heteropolysaccharides.[9−12]

Identification of L2 Conformation. The ordered structure with triple-helix conformation observed in β-(1,3)-glucan is considered the most important factor influencing cytokine-stimulating activity.[4,25] Previous studies have demonstrated that the β-(1,3)-glucan, lentinan, has antitumor activity in the triple-helix conformation but not as a single flexible chain.[24,26] Congo red reacts with triple-helix polysaccharides, which shifts the maximum absorption toward the long wavelength in solution and does not react with other polysaccharides.[27] The transition from triple-helix conformation to single coil conformation decreases the maximum absorption in Congo red−polysaccharide solution. The Congo red method was employed at NaOH concentrations ranging from 0 to 0.4 mol/L to investigate the conformation of L2. Curdlan and laminarin are typical triple-helix conformational polysaccharides. Maximum absorption wavelength indicated that the Curdlan and laminarin solutions were around 506−508 nm (Figure 2). A shift in maximum absorption wavelength from 498 to 506 nm occurred in distilled water, indicating that triple-helix polysaccharide−Congo red complexes had formed. The helix conformation was disrupted at higher

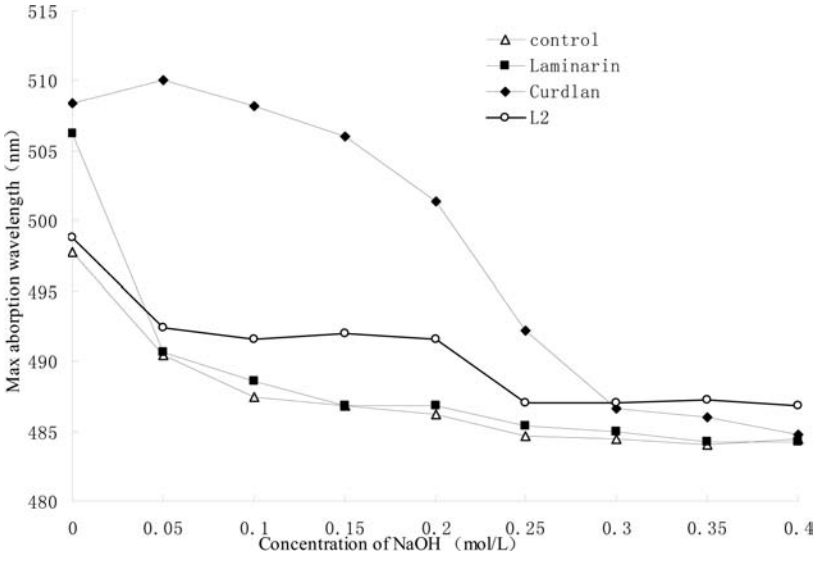

Figure 2. Helix−coil transition analysis of L2 and triple-helix conformational glucans at different concentrations of NaOH.

concentrations of NaOH, leading to a decrease in the maximum absorption wavelength. As shown in Figure 2, polysaccharide L2 with Congo red did not show the specific shift of the maximum absorption wavelength at different concentrations of NaOH, demonstrating the absence of triple-helix conformation in L2, as in the control.

Immuno-Stimulating Activities of L2. *Effects of L2 on NO, TNF-α, and IL-6 Production.* Activated macrophages secrete TNF-α, IL-6, and NO, which play important roles in fighting tumor cells and microbes. L2 increased the NO production of RAW 264.7 cells in a dose-dependent manner (Figure 3A). It had similar effects on IL-6 and TNF-α secretion

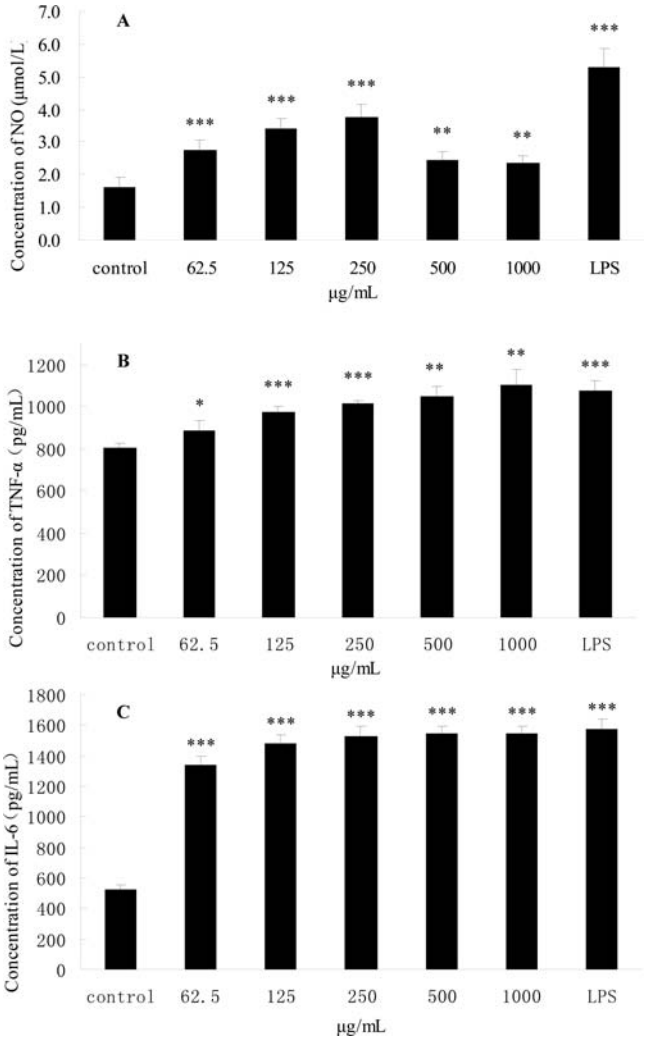

Figure 3. Effects of different concentrations of L2 on (A) NO, (B) TNF-α, and (C) IL-6 production of RAW 264.7 cells, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control (without L2). LPS (50 μg/mLl) served as the positive control. The data shown are means ± SD. The experiments were performed in triplicate, and a representative example is shown.

at L2 concentrations below 250 μg/mL (Figure 3B and C). However, when the concentration of L2 increased from 250 to 1000 μg/mL, the amount of NO decreased, and the amounts of IL-6 and TNF-α remained stable (Figure 3B and C). These results indicated the existence of the optimal dose for the stimulating of NO production, which was similar to the pattern observed when peritoneal macrophages are exposed to lentinan in vivo.[28] However, lentinan reduced the release of IL-6.[28] Taken together, these results indicated that L2 exhibits structure and immuno-modulatory properties markedly different from those of the typical triple-helix polysaccharide lentinan and other reported heteropolysaccharides derived from the fruit body of *Lentinula edodes*.[8−12,28] In the present study, for the first time, a new heteropolysaccharide lacking the triple-helix conformation was separated from the fruit body of *Lentinula edodes* and found to exhibit significant immuno-stimulating activities.

Roles of TLR2, TLR4, CR3, and Dectin-1 in L2-Induced TNF-α and IL-6 Production. It has been proposed that pattern recognition receptors (PRRs) on the immune cells play important roles in recognizing pathogen-associated molecular patterns (PAMP) derived from bacteria, fungi, and viruses. Studies have suggested that lentinan can mediate immunomodulating effects through specific immune receptors, including complement receptor 3 (CR3), TLR2, TLR4, and Dectin-1.[29−31] To determine whether TLR2, TLR4, CR3, or Dectin-1 plays a role in L2-mediated stimulation of macrophages, anti-TLR2, -TLR4, and -CR3 antibodies and the Dectin-1 inhibitor laminarin were preincubated with RAW264.7 cells for 1 h and then incubated with L2. Laminarin, a low-molecular-weight β-glucan, can bind to Dectin-1 and inhibit the immune responses associated with Dectin-1.[32] The production of TNF-α and IL-6 in RAW264.7 cells was determined. As shown in Figure 4, after anti-TLR2 and L2 incubation, the secretion of IL-6 was significantly decreased but still significantly higher than in the absence of L2 stimulation. However, the anti-TLR4, anti-CR3, and laminarin treatments were unable to inhibit the increases in TNF-α and IL-6 production (Figure 4A, C, and D). No decreased TNF-α production was observed in the corresponding anti-TLR2 group, indicating different signaling pathways for IL-6 and TNF-α production in RAW264.7 cells. These results demonstrate that TLR2 is one of the PRRs of L2 on RAW264.7 cells and the receptors like TLR4, CR3, and Dectin-1 can be excluded. Other immune receptors are also possible. The PRRs of L2 on immune cells differ significantly from those of lentinan.[29−31] Previous studies have proved that zwitterionic polysaccharide can activate immune cells through TLR2.[33] L2 was separated using DEAE cellulose elution with 0.05 mol/L NaCl solution, indicating that L2 has slight anionic charges, which may contribute to the ability of L2 to bind to TLR2 on RAW264.7 cells.

Effects of L2 on NO, TNF-α, and IL-6 Production under Different pH and Thermal Treatments. High-temperature and high sodium hydroxide concentration treatments when used on typical β-glucan from *Lentinula edodes*, called lentinan, can collapse the triple-helix conformation and lead to loss of activity.[34,35] Here, we tested the effects of different pH solvents and thermal treatments on the activity of L2. As shown in Figure 5, treating L2 with temperatures below 121 °C for 30 min produced no obvious differences in TNF-α, IL-6, and NO production in RAW264.7 cells. However, after thermal treatment at 137 °C for 30 min, L2 lost most of its ability to stimulate the production of NO, TNF-α, and IL-6. There were no obvious differences in molecular weight between treated and untreated L2, as indicated by HPGPC analysis, indicating that the chemical bonds of L2 were not broken (data not shown). These results demonstrate that the ordered conformation of L2 was disrupted by thermal treatment at 137 °C, leading to the loss of immuno-stimulating activities. The results were consistent with those of a previous study showing that the helix−coil transition was

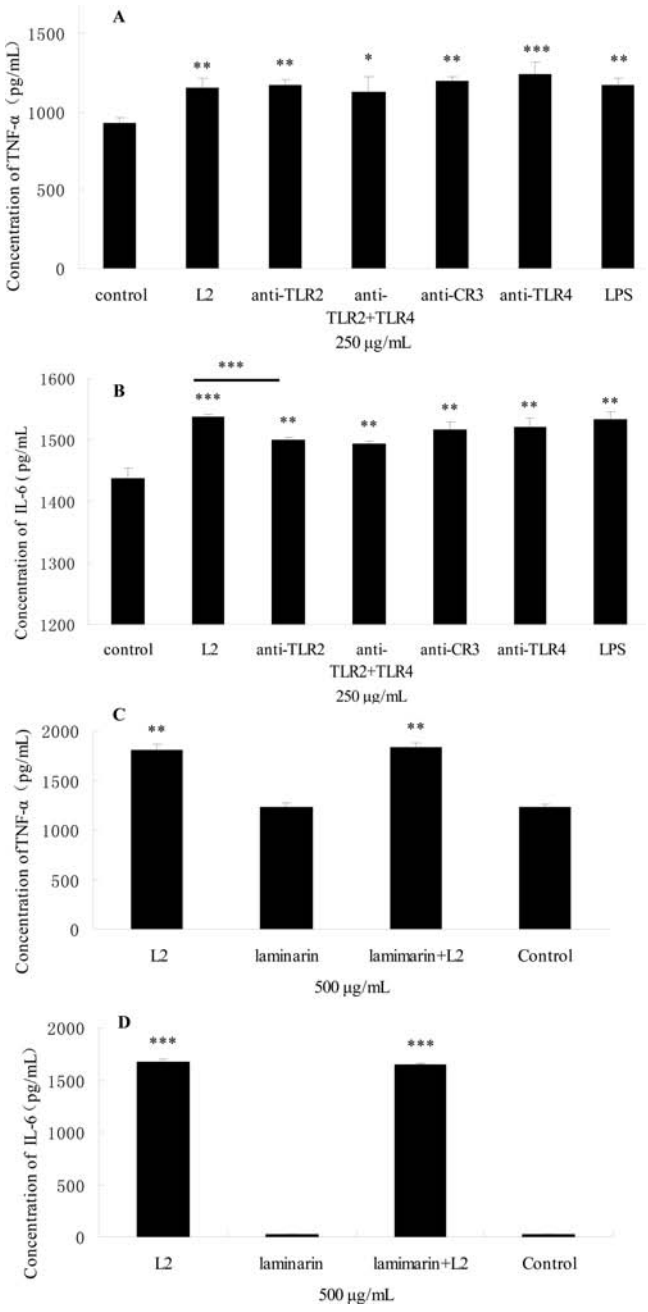

Figure 4. Roles of TLR2, TLR4, CR3, and Dectin-1 on the TNF-α and IL-6 secretion of RAW 264.7 cells. (A and B) The cells were incubated with monoclonal antibodies for 1 h and then washed extensively before stimulation with L2. (C and D) The cells were incubated with laminarin for 1 h before stimulation with L2. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control (without L2). Bars with superscripts indicate statistically significant differences from the L2 group ($P < 0.001$). The data shown are means ± SD. The experiments were performed in triplicate, and a representative example is shown.

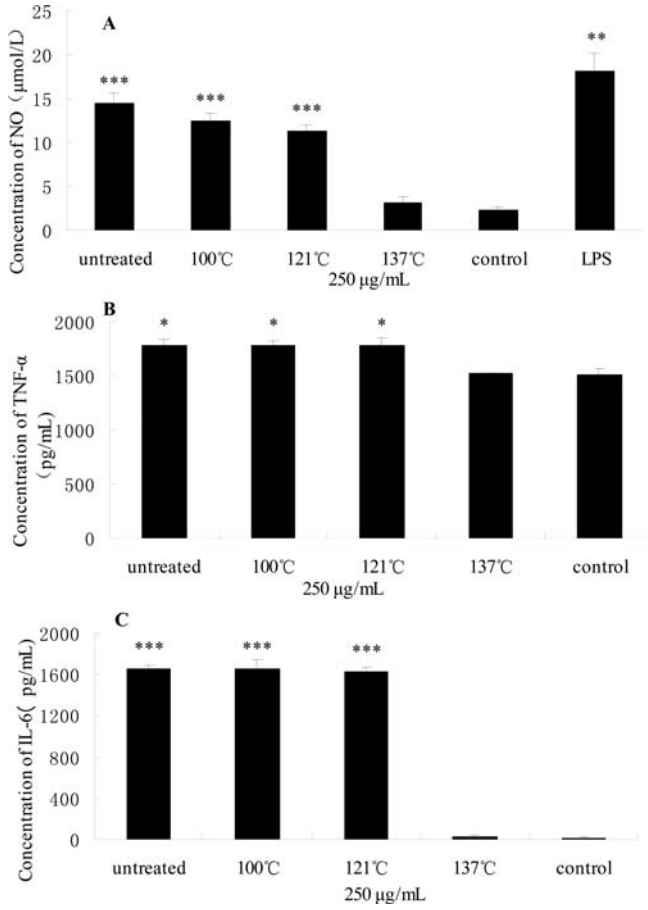

Figure 5. NO, TNF-α, and IL-6 production in RAW264.7 cells incubated with L2 for 24 h at different temperatures. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group (without L2). The data shown are means ± SD. The experiments were repeated three times, and a representative example is shown.

induced by thermal treatment at about 130−145 °C for the breakage of hydrogen bonds.[34]

The destruction of hydrogen bonds also occurred when the concentration of sodium hydroxide or pH increased.[35] As shown in Figure 6, after treatment with various phosphate buffers ranging in pH from 4.0 to 10.0, L2 was also able to stimulate NO, TNF-α, and IL-6 production, demonstrating that the active conformation of L2 remained stable across a wide range of pH levels. NO production decreased only at pH 10.0, indicating that the active conformation had been impaired. This is in accordance with the behavior of lentinan molecules, which coil randomly in pH 10 solvent.[36] These results indicated that L2 has an ordered conformation in aqueous solutions ranging in pH from 4.0 to 8.0 and that its ability to stimulate NO production is more sensitive to changes in conformation than its ability to stimulate TNF-α and IL-6 production. Furthermore, combinations of thermal and different pH treatments were also performed on L2: 121 °C for 30 min in phosphate buffers ranging in pH from 4.0 to 10.0. The results showed that L2 still retained its ability to stimulate NO, TNF-α, and IL-6 production (Figure 7). In this way, the immuno-stimulating activities of L2 were proved to be stable across wide ranges of pH and thermal processing environments. In the food industry, thermal processing is fundamental. Generally, the application of heat makes products commercially sterile. The pH and acidity of food products are usually used to determine processing requirements. The approximate pH values in food products range from 8.4 to 2.0.[37] The pH values of most major food products exceed 4.0. Thus, L2 shows potential for practical use in the food industry as a functional component.

In this study, a new heteropolysaccharide was isolated from the fruit body of *Lentinula edodes* using boiling water. This heteropolysaccharie consists of glucose (87.5%), galactose (9.6%), and arabinose (2.8%) and has an average molecular weight 26 KDa. It has an ordered conformation but not a triple-helix conformation in

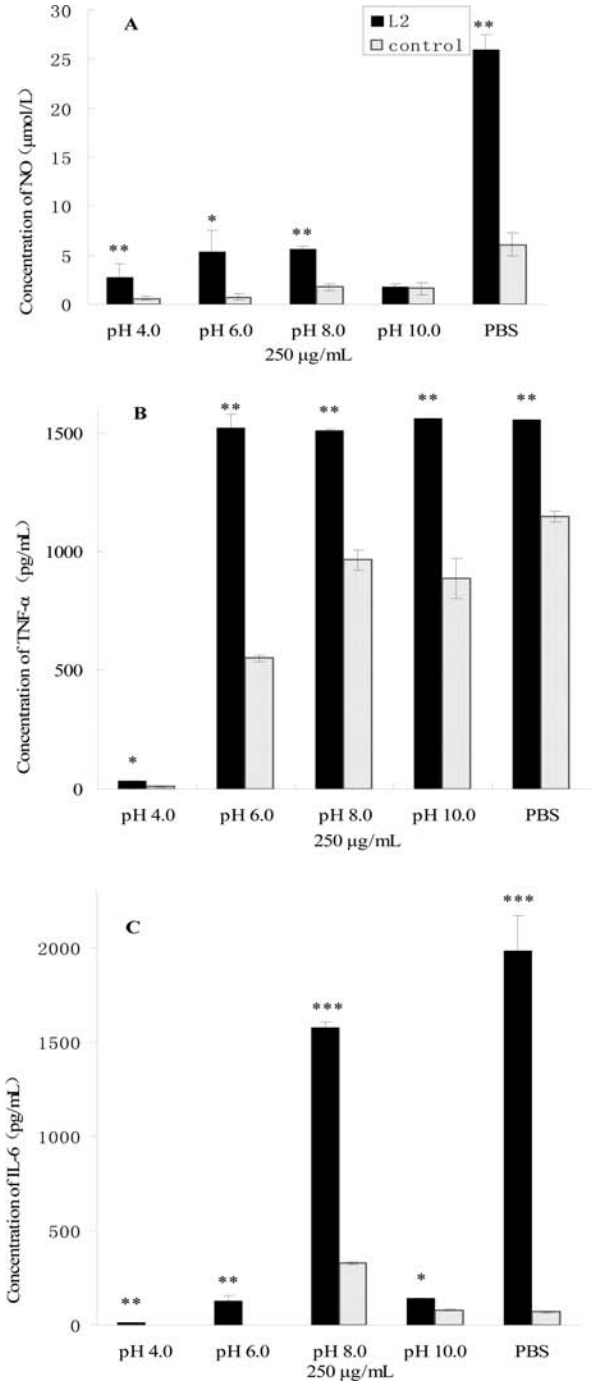

Figure 6. NO, TNF-α, and IL-6 production in RAW264.7 cells incubated with L2 for 24 h at phosphate buffers of different pH. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group (without L2). The data shown are means ± SD. The experiments were performed in triplicate, and a representative example is shown.

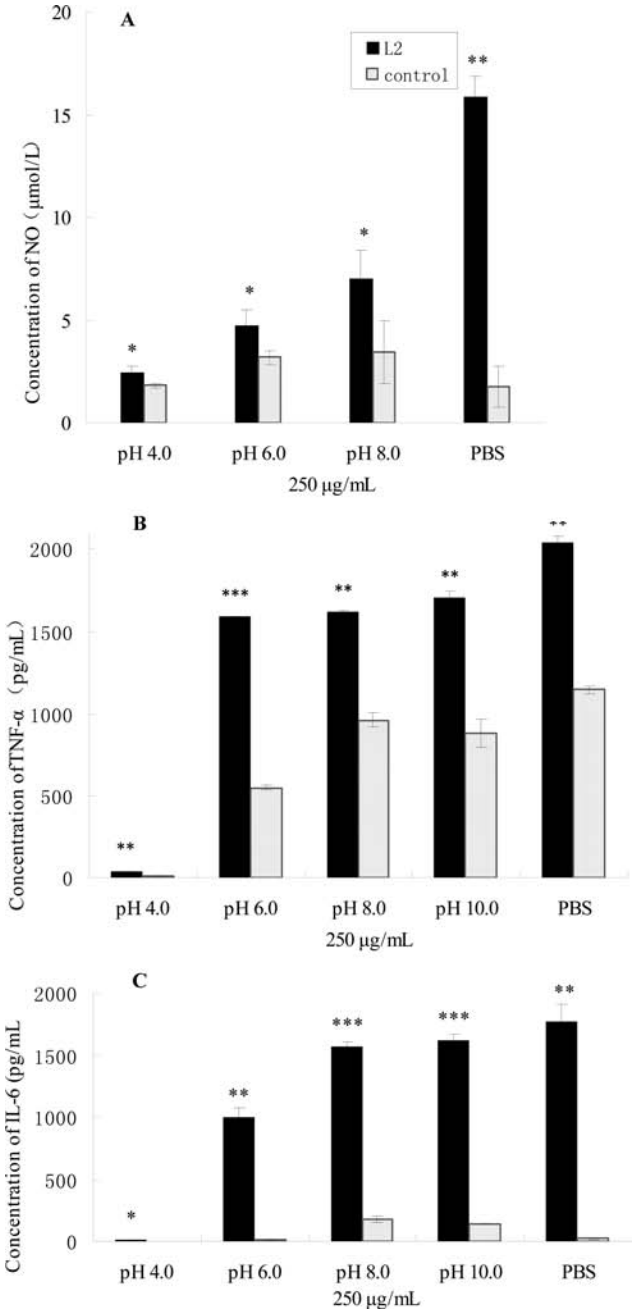

Figure 7. NO, TNF-α, and IL-6 production in RAW264.7 cells incubated for 24 h with L2 samples, which were treated at 121 °C for 30 min in phosphate buffers of different pH. *P < 0.05, **P < 0.01, ***P < 0.001 versus control groups (without L2). The data shown are means ± SD. The experiments were performed in triplicate, and a representative example is shown.

aqueous solution, and it exhibits significant immuno-stimulating activities involving TLR2. Its structure and immuno-stimulating properties were found to be markedly different from those of lentinan and other reported heteropolysaccharides derived from the fruit body of *Lentinula edodes*. The immuno-stimulating activities of this heteropolysaccharide remained stable across wide ranges of pH, from 4.0 to 10.0, and at thermal processing temperatures below 121 °C. These characteristics indicate that L2 is suitable for applications as a dietary intervention in situations where the function of the immune system needs to be stimulated. More importantly, our results demonstrate that, in addition to triple-helix β-glucan derived from mushrooms, whose antitumor and immuno-modulating activities are well recognized, this heteropolysaccharide lacking the triple-helix conformation also has immuno-modulating activities and may be used as an active component in functional foods and as a food supplement.

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Author Contributions

[§]Xu XF and Yan HD contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

TNF-α, tumor necrosis factor α; IL-6, interleukin 6; TLR, toll-like receptor; CR3, complement receptor 3; HPGPC, high performance gel permeation chromatography; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern

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