Structure elucidation and immunological function analysis of a novel β-glucan from the fruit bodies of Polyporus umbellatus (Pers.) Fries

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β-Glucans derived from various sources such as yeast cell walls and medicinal mushrooms are considered as valuable biological response modifiers for their ability to enhance the activity of immune cells, aid in wound healing and help prevent infections. We herein characterize the structure of a novel water-soluble polysaccharide (Zhuling polysaccharide, ZPS) from the fruit bodies of medicinal mushroom Polyporus umbellatus and investigate its immunobiological function. ZPS has a molecular mass of $2.27 \times 10^3$ kDa and contains >90% D-glucose as its monosaccharide constituent. On the basis of partial acid hydrolysis, methylation analysis, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy and the ideal repeating unit of ZPS is established: $(1 \rightarrow 6, 1 \rightarrow 4)$-linked β-D-glucopyranosyl backbone, substituted at O-3 position of $(1 \rightarrow 6)$-linked β-D-glucopyranosyl by $(1 \rightarrow 3)$-linked β-D-glucopyranosyl branches. ZPS consists of approximately 2930 repeating units, each contains a side chain of no more than three residues in length. Functionally, ZPS is a potent activator of B cells, macrophages and dendritic cells. Depletion of ZPS branches causes a substantial reduction in its ability not only to activate B cells in vitro but also to elicit specific IgM production in vivo. Virtually all healthy human subjects possess high-titer circulating antibodies against ZPS backbone, suggesting that ZPS epitope is shared by environmental antigens capable of eliciting adaptive humoral responses in the population.

Keywords: β-glucan / immunostimulation / lymphocyte / macrophage / structure

Introduction

β-Glucans are β-linked linear chains of D-glucose polymers with variable frequency of branches. β-Glucans isolated from different sources such as cell walls of baker’s yeast (Saccharomyces cerevisiae) and various species of mushrooms are considered as biological response modifiers (BRMs) for their ability to enhance/modulate immunity through the activation of immune cells, particularly macrophages (Bohn and BeMiller 1995; Wasser and Weis 1999; Akrami et al. 2007; Novak and Vetvicka 2008), and are widely used in aiding wound healing, help prevent infection and treatment of cancer (Morikawa et al. 1985; Babineau et al. 1994; Chen and Seviour 2007; Driscoll et al. 2009). In both animal and human studies, therapy with β-glucan preparations has provided improvements such as fewer infections, reduced mortality and stronger tensile strength of scar tissue (Browder et al. 1990; Babineau et al. 1994). For instance, zymosan, a particulate polysaccharide mixture consisting mainly of β-(1→3)/(1→6)-glucans from yeast cell walls, potentiates acute liver damage after galactosamine injection (Cui et al. 1998). β-(1→3)/(1→6)-glucans also reportedly lower low density lipoprotein cholesterol levels in humans (Naumann et al. 2006). In China and Japan, mushroom-derived extracts rich in β-glucans have been used for over 20 years as adjuncts to chemotherapy against cancers. In recent years, much effort has been spent investigating molecular mechanisms for the beneficial clinical effects of β-glucans. It has been shown that zymosan can activate macrophages, important players in wound healing after surgery or trauma, via specific binding to dectin-1 and Toll-like receptor 2 (TLR2) (Gantner et al. 2003; Sato et al. 2003; Brown 2006). In vitro, β-glucan activation of macrophages leads to the production of proinflammatory cytokines, arachidonate mobilization, protein phosphorylation and inositol phosphate formation (Sato et al. 2003).

β-Glucan polysaccharides from different sources vary in the backbone linkage as well as frequencies and linkage bonding.
in their branches, and the structural characteristics of β-glucans substantially affect their effectiveness as BRMs (Bohn and BeMiller 1995). It is of great interest to identify and characterize novel β-glucans that are more potent immune activators and exhibit better therapeutic potential in the clinic. Zhuling (the fruit bodies of Polyporus umbellatus) is a traditional Chinese medicinal herb well known for its beneficial properties in a number of conditions such as edema, scanty urine vaginal discharge and jaundice. The beneficial effects of Zhuling are largely attributed to its water-soluble polysaccharide component (Zhuling polysaccharide, ZPS), which is widely used in China in the intravenous form for the treatment of hepatitis B and certain cancers (Liu et al. 2001). We have recently found that, compared with previously well-characterized β-glucans, ZPS exhibited much stronger immunostimulatory activities towards B lymphocytes, macrophages and dendritic cells. However, the structure of ZPS is so far unclear and its structure–functional activity relationship has not been investigated. We herein characterize ZPS structure and compare its antigenicity and immunologic activities with laminarin, a typical soluble β-(1 → 3)/1(1 → 6)-glucan from the alga Laminaria digitata (Chen and Seviour 2007).

Results

Composition and linkage analysis

A water-soluble polysaccharide fraction (ZPS) was obtained from the fruit bodies of P. umbellatus (Zhuling) through a series of diethylaminoethyl-cellulose (DEAE) anion exchange cellulose and gel-permeation chromatography. The resultant ZPS showed a single and symmetrical peak on high-pressure liquid chromatography (HPLC) (Figure 1A), with an estimated molecular weight $2.27 \times 10^5$ kDa based on T-series dextran standards of 1–2000 kDa. Sugar content in ZPS was 96.8%, mostly (>90%) glucose, as determined by the phenol-sulfuric method and gas chromatography (GC) analysis (Figure 1B), respectively. Absolute configuration test revealed that all monosaccharides in the glucan were of α-configuration. Fourier transform infrared spectroscopy (FT-IR) studies on ZPS observed hydroxyl and C–H stretching vibration absorption at 3421.3 and 2925.7 cm$^{-1}$, respectively (data not shown), suggesting the presence of pyranose form glucosyl residues. Methylation analysis by GC mass spectrometry (GC-MS) produced mainly five partially methylated alditol acetates: 1,5,6-tri-acetyl-2,3,4-tri-O-methyl glucitol, 1,3,5,6-tetra-acetyl-2,4-di-O-methyl glucitol, 1,4,5-tri-acetyl-2,3,6-tri-O-methyl glucitol, 1,3,5-tri-acetyl-2,4,6-tri-O-methyl glucitol and 1,5-di-acetyl-2,3,4,6-tetra-O-methyl glucitol (Figure 1C and Table I). Correspondingly, the following five glucoside linkage forms exist in ZPS: (1 → 6)-linked glucosyl (residue A), (1 → 3,6)-linked glucosyl (residue B), (1 → 4)-linked glucosyl (residue C), (1 → 3)-linked glucosyl (residue D) and non-reducing terminal glucosyl (residue E), indicating ZPS as a branched heterogluca. The relative molar ratio of these residues in ZPS is shown in Table I.

Structural analysis

For further validation of ZPS structure, nuclear magnetic resonance (NMR) studies were carried out and the data assignment was resolved by extensive 1D and 2D NMR spectra, including $^1$H, $^{13}$C, heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum coherence (HSQC), nuclear overhauser enhancement spectroscopy (NOESY) and distortionless enhancement by polarization transfer (DEPT) (Figure 2 and Table I). The signals at δ4.50, 4.53, 4.51, 4.74 and 4.78 in the $^1$H NMR spectrum were assigned to the anomeric protons of residues A–E, respectively. The anomeric carbon data in the $^{13}$C NMR were all above δ103, with only one signal assignable at δ103.7 due to their overlap. The above data confirm that all residues in ZPS are of β-configuration. The downfield shifting resonances at δ85.0 and 79.0 in the $^{13}$C NMR spectrum, due to the α-effect of glycosidation, are ascribed to the C-3 of residues B and D and the C-4 of residue C, respectively. The downfield data at δ69.6 are ascribed to the C-6 signals of residues A and B. The O-6-substitution of these residues can be further supported by the corresponding converse signals in the DEPT spectrum (Figure 2C).

Specific linkages of glycosyl residues were determined mainly by HMBC and NOESY experiments (Figure 2 and Table I). The inter-residue HMBC correlations from H-1 of residue A (A) to C-6 of residue B (B) and H-6 of residue B to C-1 of residue A, in collaboration with the inter-residue NOESY effects of H-1 (A) with H-6 (B), established the linkage of residues C-1 of residue A to the O-6 position of residue B. The inter-residue HMBC correlations from H-1 of residue B to C-4 of residue C and H-4 of residue C to C-1 of residue B, in collaboration with the inter-residue NOESY effects of H-1 (B) with H-4 (C), established the linkage of residues C-1 of residue B to the O-4 position of residue C. The inter-residue HMBC correlations from H-1 of C to C-6 of residue A and the H-6 of residue A to C-1 of residue C, in collaboration with the inter-residue NOESY effects of H-1 (C) with H-6 (A), established the linkage of residues C-1 of residue C to O-6 position of residue A. The observed HMBCs from H-1 (D) to C-3 (B), together with the NOESY effect of H-1 (D) with H-3 (B), constructed the branch substitution located at C-3 of residue B. Similarly, the HMBCs from H-1 (E) to C-3 (D), in combination with the corresponding inter-residue NOESY effects of H-1 (E) with H-3 (D), revealed that the terminal residue E attached at C-3 of residue D.

In comprehensive composition analysis, methylation analysis and NMR experiments, it can be concluded that ZPS is a glucan with a backbone structure of (1 → 6, 1 → 4)-linked β-D-glucopyranosyl (glcp) residues, which is substituted at the O-3 position of (1 → 6)-linked β-D-glucopyranosyl by side chains of (1 → 3)-linked β-D-glucopyranosyl residues and non-reducing end β-D-glucopyranosyl residues. The repeating structure unit of ZPS is illustrated in Figure 3. ZPS structure deduced above can be further confirmed by the results of partial hydrolysis analysis. Mild hydrolysis of branched polysaccharides with trifluoroacetic acid (TFA) can trim off some or most of their side chains while leaving the backbone intact, treatment with 0.8 N TFA almost completely got rid of ZPS side chains (see below). Methylation analysis by GC-MS revealed that 0.8 N TFA-treated ZPS (ZPS-0.8N) is composed of 1,6-linked-glp, 1,3,6-linked-glp, 1,3-linked glp, 1,4-linked glep and 1,4-linked glep in the molar ratio of
5.06:0.38:0.53:6.31:8.40. Compared with untreated ZPS (Table I), the molar ratio of 1,6-linked-glcp and 1,4-linked-glcp in ZPS-0.8N raised significantly, indicating the backbone location for these residues. Conversely, the molar ratio of 1,3,6-linked-glcp and 1,3-linked glcp in ZPS-0.8 N reduced sharply, confirming that 1,3-linked glcp spreads in the side chains and also that ZPS branches at the O-3 position of 1,3,6-linked-glcp residues.

HPLC analysis of ZPS-0.8 N, using a Waters ultra-hydrogel linear GPC column of 7.8 nm × 300 nm, revealed its molecular mass as $1.41 \times 10^3$ kDa, less 860 kDa than untreated ZPS. It can thus be estimated that ZPS consists of $\sim 8800$ glucopyranosyl residues in its backbone (2930 repeating units) and at least 5200 residues in branches. This gives a side chain/backbone ratio of 0.61, which is very close to the figure calculated using the GC-MS data shown in Table I. In order to further characterize ZPS side chains, a periodate oxidization and NaBH$_4$ reduction method was employed to selectively break the (1 → 4) and (1 → 6)-linked glucopyranosyl linkages. The resultant product was dialyzed against distilled water, and the dialysate out of

Fig. 1. HPLC, GC and methylation linkage analysis. ZPS was analyzed by HPLC (A). Sample solution was injected with water as the mobile phase at a flow rate of 1 mL/min. Monosaccharide composition of ZPS was determined by GC (B). ZPS was hydrolyzed using 2 N TFA and the resultant monosaccharides converted into alditol acetates, followed by GC-MS (C). Five glucosidic linkage forms (residues A–E) in the total ion chromatogram are indicated.
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Residues Methylated sugars Type of linkage Molar ratio Location
A 2,3,4-Me3-Glc 1,6-linked Glcp 9.25 Backbone
B 2,4-Me2-Glc 1,3,6-linked Glcp 8.29 Backbone
C 2,3,6-Me3-Glc 1,4-linked Glcp 9.35 Backbone
D 2,4,6-Me3-Glc 1,3-linked Glcp 7.90 Side chain
E 2,3,4,6-Me4-Glc Terminal Glcp 8.65 Side chain

*The result was obtained using a DB-5 GC-MS column.
†All the sugar residues were primarily identified by their MS spectrum and further confirmed by their retention time relative to 2,3,4,6-Me4-Glc.
‡The side chain/backbone molar ratio is calculated as (7.9 + 8.65) / (9.25 + 8.29 + 9.35) = 0.615, which is very close to that (0.61) calculated using the molecular mass of ZPS backbone (1410 kDa) and side chains (860 kDa).

Stimulatory activities to B lymphocytes and macrophages

For function analysis, ZPS was compared with laminarin and dextran (α-1,6-glucan, as a negative control) for the ability to activate B cells and macrophages. Mouse peritoneal macrophages produced significantly more interleukin (IL)-1β in response to stimulation with ZPS than laminarin (Figure 4A). ZPS was also more potent than laminarin in inducing proliferative responses of murine splenocytes (Figure 4B). To assess the immunostimulatory activity of ZPS in further detail, B- and T-lymphocytes were fractionated by magnetic sorting from mouse splenocytes and tested in proliferation assays against ZPS, laminarin, bacterial lipopolysaccharide (LPS), a B cell mitogen) and concanavalin A (ConA) (a T cell mitogen). T cells did not respond to stimulation with either ZPS or laminarin. Purified splenic B cells proliferated well in response to stimulation with ZPS, but poorly to laminarin (Figure 4C). To exclude the possibility that our ZPS preparations had been contaminated with LPS, polymixin B (PMB, an effective LPS inhibitor) was employed to inhibit the activity of possible LPS contaminant in ZPS. LPS, but not ZPS, was susceptible to PMB inhibition in these assays (Figure 4D), implying that LPS contamination in ZPS, if any, was negligible.

Effect of side chain depletion on ZPS activity

It has been suggested that the side chains of polysaccharides substantially affect their immunological activities. ZPS was treated with 0.1, 0.5 and 0.8 N TFA at 90°C for 1 h, followed by dialysis against distilled water. Subsequent methylation analysis on TFA-treated ZPS samples (namely ZPS-0.1 N, ZPS-0.5 N and ZPS-0.8 N) by GC-MS confirmed the gradual depletion of the side chains with increasing TFA concentration, which is further collaborated by HPLC results showing the molecular mass of ZPS-0.1 N, ZPS-0.5 N and ZPS-0.8 N as 2.06 × 10^3, 1.61 × 10^3 and 1.41 × 10^3 kDa, respectively. All TFA-treated samples (including ZPS-0.8 N) were more potent than laminarin at inducing tumor necrosis factor (TNF)-α production by murine macrophages in vitro (Figure 5A), indicating that TFA treatment had a relatively limited effect on the macrophage-stimulating activity of ZPS. On the other hand, TFA treatment substantially reduced its B cell-stimulating activity, as ZPS-0.8 N and ZPS-0.5 N were significantly less potent in eliciting B cell proliferation in vitro (Figure 5B). ZPS, ZPS-0.1N, ZPS-0.5N and ZPS-0.8N were further compared for ability to elicit IgM production in adult BALB/c mice following i.p. administration. Apparently, the treatment of ZPS with increasing concentration TFA caused gradual reduction in its immunogenicity (Figure 5C).

Specific recognition of ZPS by human circulating Abs

Humans are known to possess circulating antibodies (Abs) against β-glucans such as laminarin (Chiani et al. 2009). By using polysaccharide-based enzyme-linked immunosorbent assay (ELISA) systems, we analyzed serum samples from healthy human subjects of different blood groups for Abs against ZPS and laminarin. Interestingly, titers of IgM, IgG and IgA against ZPS were much higher than that for laminarin (Figure 6A–C). The presence of ZPS-specific Abs was irrespective of the blood group of the donors. Dextran and mannann were poorly recognized by human serum Abs, in line with our previous reports (Dai et al. 2009; Dai and Gao 2011). An equal proportion mixture of sera from 30 healthy human adults was titrated against ZPS and TFA-treated ZPS derivatives in ELISAs. Although ZPS-0.8 N seemed less well recognized by human IgM when compared with ZPS, ZPS-0.1 N or ZPS-0.5 N, the difference was not statistically significant (Figure 6D), suggesting that anti-β-glucan human circulating Abs mainly recognize the backbone structure rather than side chains of ZPS.

Discussion

Following a series of chemical and analytical procedures including partial acid hydrolysis, methylation analysis, HPLC, FT-IR and NMR studies, we have successfully determined the structure of ZPS, a novel β-glucan polysaccharide from P. umbellatus. ZPS has an ideal repeating unit of (1→3)-β-D-glucopyranosyl by (1→6)-D-glucopyranosyl backbone, substituted at O-3 position of (1→6)-linked β-D-glucopyranosyl by (1→3)-linked β-(1→3)-D-glucopyranosyl branches (Figure 3). It is consisted of ~2930 repeating units, each contains a side chain of no more than three residues in length.

This β(1→6, 1→4)(1→3)-glucan structure is unique among the known naturally produced β-glucans, most polysaccharides isolated from medicinal mushrooms possess a β(1→3)-glucan backbone (Bohn and BeMiller 1995; Akramiene et al. 2007; Chen and Seviour 2007). Interestingly, ZPS exhibits more potent stimulatory activities against macrophages than the β(1→3)/(1→6)-D-glucans represented by laminarin. Additionally, it is capable of efficiently activating dendritic cells (DCs) in vitro (data not shown). These results have important implications for our understanding of the molecular mechanisms behind the therapeutic effect of β-glucan BRMs. We postulate that ZPS may exert its immunoenhancing effects via three different, but not mutually exclusive, pathways: (i) as a macrophage modulator, influencing tissue
Fig. 2. 1D and 2D NMR. $^1$H-$^1$C HMBC (A), HSQC (B) and 1D DEPT (C) NMR spectra of ZPS were determined in D$_2$O at 25°C using a Bruker AM 500 spectrometer with a dual probe in the FT mode. Each cross-peak corresponds to a C-H pair. $^1$H was measured in 500 MHz and $^1$C in 125 MHz. TMS was used as an external standard for the $^1$C NMR spectrum and D$_2$O as internal standard for the $^1$H NMR spectrum.
macrophages that are key players in wound healing and also in innate immunity against microbial invasion; (ii) as a professional antigen-presenting-cell enhancer, promoting antigen processing and presentation by DCs and macrophages during initiation of adaptive immune responses; (iii) as a B cell booster, assisting the activation/proliferation of antigen-specific B cells though TLR4 and other co-receptors during humoral responses.

In flow cytometry analysis, fluorescein isothiocyanate (FITC)-conjugated ZPS was able to bind selectively to murine B cells and macrophages, but not T cells (Supplementary data, Figure). Both ZPS and laminarin effectively inhibited FITC-Zymosan uptake by macrophages in vitro (data not shown), indicating that they share the same phagocyte receptors, which include dectin-1, complement receptor-3, TLR2 and scavenger receptors (Brown and Gordon 2003; Sato et al. 2004; Brown 2006). Co-precipitation experiments identified several novel ZPS-binding proteins in the lysate of murine peritoneal macrophages and a human monocytic leukemia line THP-1 cells (Dong HL et al., manuscript in preparation). Ongoing study in our laboratory will aim to identify additional ZPS receptors on murine immune cells.

Although the ability for branched β-glucans to induce specific IgM production in vivo has previously been noted (Shao et al. 2004), studies delineating molecular mechanisms for direct interaction of β-glucans with B cells are rather limited. Dectin-1 is known to be expressed by human B lymphocytes (Willment et al. 2005; Brown 2006; Gringhuis et al. 2012), but not by murine B220+ B cells (Taylor et al. 2002). Thus, murine B cells would have to employ a different surface receptor(s) for β-glucan recognition. The unique ZPS backbone structure may ensure better interaction with B cell antigen receptors and/or pattern recognition receptors. Additionally, high branching frequency of ZPS could lead to better cross-linking of surface molecules. Depletion of the β-(1→3)-linked branches in ZPS by TFA (0.5 and 0.8 N) treatment did not only reduce its ability to activate murine B cells in vitro (Figure 5B) but also render it unable to elicit specific IgM responses in mice (Figure 5C).

The presence of much higher titer IgM, IgA and IgG Abs specific for ZPS than laminarin in the sera of healthy human subjects is intriguing. Presumably, such Abs were elicited by environmental microorganisms, as β-glucans are cell wall components of commensal bacteria and fungi and considered one of the major pathogen-associated molecular patterns (Brown and Gordon 2003). Strong recognition of ZPS-0.8 N by human circulating Abs suggests that the target ZPS epitope(s) is localized within its (1→6, 1→4)-linked β-d-glucopyranosyl backbone, which is not shared by the previously known β-(1→3)/(1→6) glucans. Our results also raise concerns with regards to i.v. administration of ZPS and other β-glucan products, as they could form immune complexes with pre-existing serum Abs in humans. Immune complexes are known to be effective activators of macrophages and can initiate immunopathological reactions when deposited in tissues. The antigenicity and structure–function relationship of ZPS merit further investigation.

Table II. 1H NMR and 13C NMR chemical shifts of ZPS recorded in D2O at 25°C

<table>
<thead>
<tr>
<th>Glycosyl residues</th>
<th>H-1/C-1</th>
<th>H-2/C-2</th>
<th>H-3/C-3</th>
<th>H-4/C-4</th>
<th>H-5/C-5</th>
<th>H-6a,H-6b/C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, β-d-glucopyranosyl</td>
<td>4.50/103.7</td>
<td>3.30/72.3</td>
<td>3.48/76.0</td>
<td>3.46/70.0</td>
<td>3.66/75.0</td>
<td>3.86, 4.21/69.6</td>
</tr>
<tr>
<td>B, β-(1→3)β-d-glucopyranosyl</td>
<td>4.53/103.7</td>
<td>3.51/72.5</td>
<td>3.74/85.0</td>
<td>3.46/70.1</td>
<td>3.66/75.1</td>
<td>3.86, 4.21/69.6</td>
</tr>
<tr>
<td>C, β-(1→4)β-d-glucopyranosyl</td>
<td>4.51/103.7</td>
<td>3.30/72.3</td>
<td>3.48/75.9</td>
<td>3.64/79.0</td>
<td>3.62/75.0</td>
<td>3.72, 3.82/61.4</td>
</tr>
<tr>
<td>D, β-(1→3)β-d-glucopyranosyl</td>
<td>4.74/103.7</td>
<td>3.54/73.5</td>
<td>3.74/85.0</td>
<td>3.52/68.1</td>
<td>3.43/75.5</td>
<td>3.72, 3.90/61.4</td>
</tr>
<tr>
<td>E, β-(1→6)β-d-glucopyranosyl</td>
<td>4.78/103.7</td>
<td>3.34/72.1</td>
<td>3.52/75.0</td>
<td>3.45/70.0</td>
<td>3.41/75.5</td>
<td>3.72, 3.88/61.4</td>
</tr>
</tbody>
</table>

Values of 13C chemical shift were recorded with reference to TMS as an external standard.

**Conclusions**

ZPS, an effective BRM widely used in clinic, is a novel β-glucan of (1→6, 1→4)-linked β-d-glucopyranosyl backbone substituted at O-3 position of (1→6)-linked β-d-glucopyranosyl by (1→3)-linked β-d-glucopyranosyl branches. This unique β-glucan is a potent activator of B cells and macrophages, and depletion of the branches causes substantial reduction in its stimulatory activity against B cells. All healthy human subjects possess high-titer circulating antibodies against glyco-epitope(s) of ZPS, which is presumably shared by environmental agents.

**Materials and methods**

**Materials and general chemical methods**

Dried fruit bodies of *P. umbellatus* were collected in Beijing, China, in September 2008 and the specimens were kept in the herbarium of Peking University Modern Research Centre of Traditional Chinese Medicines. Sepharose CL-6B and DEAE cellulose were purchased from GE (Fairfield, CT). Standard monosaccharides, T-series dextran, TFA, dimethyl sulfoxide, laminarin, mannan and LPS were from Sigma (St Louis, MO). All chemical reagents were of grade AR grade.

The UV-Vis absorption spectrum was recorded with a Shimadzu MPS-2000 spectrophotometer. GC was performed on an Agilent 6890N instrument equipped with a HP-5 column (30 m × 0.25 mm × 0.25 μm) and detected with a flame ionization detector, the column temperature was increased from 170–215°C at a rate of 2°C/min then hold on for 5 min. GC-MS was measured on a Finnigan Trace GC-MS instrument coupled with a DB-5 column (30 m × 0.25 mm × 0.25 μm) and at temperatures programmed from...
160–250°C in a rate of 5°C/min and then held for 17 min. The FT-IR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm⁻¹.

*Extraction and isolation of polysaccharides*

The fruit bodies of *P. umbellatus* (1.0 kg) were extracted with 3 L of 95% EtOH at 100°C for 1.5 h to remove lipid. The supernatant was removed, and the residue was extracted with distilled water at 100°C three times (2 L × 3; 1.5 h each time). After centrifugation at 3600 × g for 20 min, the supernatant was concentrated 10-fold, and precipitated with 95% ethanol (1:4, v/v) at 4°C for 12 h. After centrifugation, the precipitate was suspended in distilled water to remove protein. A portion of the crude polysaccharide (8 g) was dissolved in water (100 mL) and loaded on a DEAE-52 cellulose chromatography column (5.0 × 70.0 cm), followed by elution with a 10-step gradient of 0–2 M sodium chloride. Guided by the colorimetric total carbohydrate test using the phenol-sulfuric acid method, the water eluting fraction was collected, dialyzed, lyophilized and further purified by Sepharose CL-6B (2.6 × 100 cm) gel-permeation chromatography eluted with water to obtain a purified ZPS.

*Fig. 3.* Putative ZPS structure. The repeating structure unit of ZPS is illustrated by the line drawing (A), while specific linkages of glycosyl residues in ZPS backbone and branches are further illustrated in (B) and (C). ZPS is consisted of ~2930 repeating units in total, each unit contains a (1 → 3)-linked glucopyranosyl side chain of no more than three residues in length.

*Determination of homogeneity and molecular weight*

The homogeneity and molecular weight of ZPS were determined by HPLC on an Agilent 1100 system equipped with TSK-GEL G4000PWXL column, or a Waters GPC apparatus with a 7.8 mm × 300 mm Ultra-hydrogel linear column, and evaporative light scattering detector. Sample solution (1 mg/mL, 10 μL) was injected with water as the mobile phase at a
flow rate of 1 mL/min. The linear regression was calibrated with T-series dextran standards (MW 1.27, 5.2, 10, 40, 100 and 2000 kDa). For identification and quantification of ZPS monosaccharides, 10 mg ZPS was hydrolyzed in 2 M TFA at 100°C for 2 h. The resultant monosaccharides were converted into alditol acetates as described by Jones and Albersheim (1972) and Oades (1967) and then analyzed by GC. 2-butanol described by Gerwig et al. (1979).

Methylation analysis
ZPS (10 mg) was methylated three times as described by Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of OH bands (3200–3700 cm$^{-1}$) in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated as described by Sweet et al. (1975). The partially methylated alditol acetates were analyzed by GC-MS.

Periodate-oxidization and NaBH$_4$ reduction reaction
This was the same as that described by Goldstein et al. (1965) and Hay et al. (1965). Briefly, ZPS (1 mg/mL) and NaIO$_4$ (0.03 M) were dissolved in acetate buffer at 4°C for 72 h, then excess NaIO$_4$ was destroyed with ethylene glycol for 2 h, followed by dialysis against distilled water over night. The oxidation product was then treated with NaBH$_4$ (3 mg/mL) for 3 h, made pH 7 with 10% aqueous HOAc, and dialyzed against water.

NMR studies
ZPS (50 mg) was dried in a vacuum over P$_2$O$_5$ for 72 h, and then exchanged with deuterium by lyophilizing with D$_2$O for three times. The deuterium-exchanged polysaccharide was put in a 5-mm NMR tube and dissolved in 1.0 mL of 99.96% D$_2$O. All the 1D (DEPT) and 2D (HMBC, HSQC and NOESY) NMR spectra were obtained with a Bruker AM 500 spectrometer with a dual probe in the FT mode at room temperature. Tetramethylsilane (TMS) was used as external standard for the $^{13}$C NMR spectrum, and D$_2$O was used as internal standard for the $^1$H NMR spectrum.

Preparation of mouse splenocytes, splenic T, B and peritoneal macrophages
Female BALB/c mice of 8–10 weeks were purchased from the Experimental Animal Division of Peking University Health Sciences Center, Beijing, China. All animals were maintained in the animal facility of the Department of Immunology. All cells were cultured in complete R10 medium: RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), penicillin/streptomycin (100 U/mL), L-glutamine (2 mM) and 2-mercaptol ethanol ($5 \times 10^{-5}$ M). For purification of splenic B and T cells, mouse splenocytes resuspended in R10 at $10^7$ cells/mL were plated into 90-mm tissue culture dishes and incubated for 4 h at 37°C in a CO$_2$ incubator. The non-adherent cells were collected, washed twice in phosphate buffered saline (PBS), then

![Fig. 4. Immunostimulatory activities of ZPS. BALB/c mouse peritoneal macrophages were stimulated with, or without (medium), dextran (Dex, 30 μg/mL), laminarin (30 μg/mL), ZPS (30 μg/mL) or LPS (10 μg/mL) for 48 h. The culture supernatant was then harvested and analyzed, in triplicate wells, for IL-1β by using quantitative ELISA. The results are expressed as mean IL-1β concentration (pg/mL) ± SD of the triplicate wells (A). Freshly prepared BALB/c mouse splenocytes were stimulated with ZPS, laminarin or dextran of indicated concentrations in standard 72 h proliferation assays (B). In (C), freshly fractionated splenic B (solid bars) and T (open bars) cells were stimulated with, or without (medium), dextran, laminarin or ZPS (30 μg/mL), LPS (10 μg/mL) and ConA (3 μg/mL) were included as controls. In a parallel experiment, mouse splenocytes were stimulated with, or without (medium), ZPS (30 μg/mL), dextran (30 μg/mL) or LPS (10 μg/mL) in triplicate wells in the presence, or the absence, of PMB (D). $^3$H-TdR was added to the cultures for the last 8 h of incubation and then $^3$H-TdR incorporation (CPM) of each well counted. The results are expressed as mean stimulation indices, calculated using the “cell and medium only” wells as reference, ±SD of triplicate wells. * P < 0.05; ** P < 0.01; *** P < 0.005.
incubated with MACS magnetic microbeads coated with rat anti-mouse CD19 mAb (Miltenyi Biotec, Germany) for 30 min at 4°C. The labeled cells were applied to an MACS separation column. The effluent was collected as T cells, and then B cells were flushed out of the column. Purity of the resultant B and T cells was greater than 90% as assayed by flow cytometry analysis. BALB/c mice were sacrificed three days after i.p. treatment with 3% thioglycollate. Peritoneal macrophages were harvested by peritoneal lavage using ice-cold Ca2+- and Mg2+-free PBS.

Proliferation assays

Freshly prepared splenocytes (4 × 10^5), T cells or B cells (2 × 10^5) were cultured in flat-bottom 96-well plates (Nunc, Denmark) in a volume of 200 µL/well with different concentrations of LPS, ZPS, laminarin or dextran in the presence or the absence of PMB. The cultures were incubated at 37°C and 5% CO2 for 3 days. In the last 8 h of incubation, 3H-thymidine (3H-TdR, 0.2 µCi/well) was added into each well. The cells were then harvested, using a 96-well plate harvester (Tomtec, Hamden, CT), onto fiberglass filters and radioactivity on the filter was counted in a MicroBeta Trilux LSC counter (EG&G Wallac, NORTON, OH).

Cytokine assays

The concentration of TNF-α and/or IL-1β in the culture supernatant was determined using ELISA kits (Bio Legend, San Diego, CA) following the manufacturer’s instructions. Standard curves were established using mouse recombinant TNF-α and IL-1β and the assay detection limit was 7.8 pg/mL.

Blood samples and polysaccharides-based ELISA

Serum samples were collected from 30 healthy volunteers of both sexes between 20 and 50 years of age. Distribution of the blood groups among these volunteers is: A (n = 7), B (n = 10), O (n = 7), AB (n = 6). Specific antibodies to polysaccharides were measured by ELISAs as described previously (Dai et al. 2009; Dai and Gao 2011). Detection of IgM, IgG or IgA was carried out using goat anti-human Abs coupled to horseradish peroxidase (Southern Biotechnology Associates Inc., Birmingham, AL). The reaction was developed with 100 µL of O-phenylenediamine (OPD, Sigma) for ~5 min and stopped with 100 µL of 3 M H2SO4. Optical density
(OD) was measured at 492 nm in an ELISA spectrophotometer (Titertek Multiscan Plus MK II; ICN Flow Laboratories, Irvine, UK).

Statistical analysis
All experiments were repeated at least three times. Results are presented as the mean ± standard error of the mean. Comparison of the data was performed using the Student’s t-test. Significance was defined as a P-value of <0.05.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

Abbreviations
Ab, antibody; BRM, biological response modifier; ConA, concanavalin A; DC, dendritic cell; DEAE, diethylaminoethyl-cellulose; DEPT, distortionless enhancement by polarization transfer; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FT-IR, fourier transform infrared spectroscopy; GC-MS, gas chromatography mass spectrometry; Glc, (1→6,1→4)-linked β-D-glucopyranosyl; HMBC, heteronuclear multiple-bond correlation; HPLC, high-pressure liquid chromatography; HSQC, heteronuclear single-quantum coherence; ³H-TdR, ³H-thymidine; IL, interleukin; LPS, Lipopolysaccharide; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; OD, optical density; OPD, O-phenylenediamine; PBS, phosphate buffered saline; PMB, polymyxin B; TFA, trifluoroacetic acid; TLR, Toll-like receptor; TNF, tumor necrosis factor; TMS, tetramethylsilane; ZPS, Zhuling polysaccharide.

References


