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Structural characterization of an active polysaccharide from *Phellinus ribis*

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Abstract

A water-soluble polysaccharide named as PRP was isolated from the fruiting bodies of *Phellinus ribis* by hot water extraction, DEAE-cellulose and Superdex 30 column chromatography. Its structural characteristics were investigated by FT-IR, NMR spectroscopy, GLC-MS, methylation analysis, periodate oxidation and Smith degradation. Based on the data obtained, PRP was found to be a β-D-glucan containing a (1 → 4), (1 → 6)-linked backbone, with a single β-D-glucose at the C-3 position of (1 → 6)-linked glucosyl residue every eight residues, along the main chain. The glucan has a weight-average molecular weight of about 8.59 kDa by HPGPC determination using dextran samples as the standards. Preliminary activity tests *in vitro* revealed that PRP could stimulate the proliferation of spleen lymphocyte.

Keywords: *Phellinus ribis*; D-Glucan; Structure; Activity

1. Introduction

Polysaccharides and polysaccharide-protein complexes have been found as common polymers in fungi and received considerable attention in recent years because of their anti-tumor and immunoregulating activities (Ooi & Liu, 2000; Wasser & Weis, 1999). A number of fungal glucans such as krestin, lentinan and schizophyllan are widely used as non-specific modulators of immune system, e.g., as adjuvants in cancer treatment, with low toxicity and less side effects in clinic (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985).

Phellinus ribis is a kind of fungi belonging to *Phellinus* genus, *Hymenochaetaceae* family. The acidic polysaccharide isolated from *Phellinus linteus* enhanced cell-mediated immunity, and the protein-bound polysaccharide had a direct anti-tumor effect (Kim, Choi, Lee, & Park, 2004; Li et al., 2004). The neutral polysaccharide from *P. linteus* showed hypoglycemic effect, and decreased total cholesterol and triacylglycerol (Kim et al., 2001). Extract of *Phellinus conchatus*, *Phellinus baumii* and *Phellinus rimosus* showed cytotoxic, free radical scavenging and antihepatotoxic activity, respectively (Ajith & Janardhanan, 2002; Ren, Liu, Zhu, Yang, & Fu, 2006; Shon, Kim, & Sung, 2003).

As a Chinese folk medicine, the hot water extract of *P. ribis* has been used for treating pharyngitis and enhancing immunity. No report on the polysaccharides of *P. ribis* was found till now. The present paper reports the isolation and chemical characterization of a β-glucan from the hot water extract of *P. ribis*, as well as its spleen lymphocyte proliferation stimulating activity *in vitro*.

2. Experimental

2.1. Materials

P. ribis was collected from mountain area in Jinan city, Shandong Province, China in October 2005. The voucher

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specimen of this fungus is deposited in the School of Pharmaceutical Sciences, Shandong University, Jinan, China. The fruiting bodies of *P. ribis* were air-dried in shade and were broken into multiple pieces with a mill. DEAE-cellulose 23 SS was from Serva (Heidelberg, Germany) and Superdex 30 was purchased from Amersham Biosciences (Uppsala, Sweden).

2.2. General methods

Optical rotation was measured at 20 °C with a WZZ-1 polarimeter. UV–Vis absorption spectra were recorded with a Unico™ UV-2102PC spectrophotometer. The FT-IR spectra (KBr pellets) were recorded on a Nicolet Nexus 470 FT-IR spectrophotometer. Total carbohydrate content was determined by the phenol-sulfuric acid method using D-glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined according to an *m*-hydroxyldiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991). Protein was measured by the Folin-phenol method using bovine serum albumin as standard (Lowry, Rosebrough, & Farr, 1951).

2.3. Extraction and fractionation of polysaccharide

Dried crushed fruiting bodies (1.5 kg) of *P. ribis* were extracted with distilled water (6 L) at 90 °C every 6 h for four times, filtered through gauze and centrifuged to remove water-insoluble materials. The aqueous extract was concentrated at 55 °C in vacuum and treated with four volumes of ethanol at 4 °C overnight. The resulting precipitate was collected, dissolved in water and deproteinated by a combination of trypsin enzymolysis and Sevag method. The crude polysaccharide fraction (41.5 g) was obtained through precipitation with four volumes of ethanol and desiccation in vacuum. The crude polysaccharide was redissolved in water (0.5 L), and further fractionated by precipitation with ethanol (0.5 L). After centrifugation, the supernatant was treated by adding another three volumes of ethanol at 4 °C. The resulting precipitate was dried at 50 °C in vacuum and a grayish power (named TPRP, 19.3 g) was obtained. The TPRP was applied to DEAE-cellulose column (4.5 × 30 cm), eluted with water, followed by 0.05 M NaCl, 0.1 M NaCl and 0.2 M NaCl, respectively. Based on the colorimetric test for total carbohydrate by the phenol-sulfuric acid method, the fraction eluted with water was further purified on a Superdex 30 column (1.6 × 80 cm) eluted with 0.05 M NaCl. The main fraction was collected, dialyzed and lyophilized to get a white purified *P. ribis* polysaccharide (PRP, 520 mg).

2.4. Homogeneity and molecular weight determination

The homogeneity and molecular weight of PRP was determined by high-performance gel-permeation chromatography (HPGPC) on a Waters 515 instrument equipped with a Ultrahydrogel 250 column (7.8 × 300 mm) and a Waters 2410 Refractive Index Detector (RID). 20 μl of sample solution (0.5% PRP solution) was injected in each run, with 0.05 M Na_2SO_4 as the mobile phase at a flow rate of 0.8 ml/min. The HPGPC system was precalibrated with T-series Dextran standards (T-10, T-20, T-40, T-70, T-110 and T-500).

2.5. Monosaccharide composition analysis

PRP (10 mg) was hydrolyzed in 2 M trifluoroacetic acid (TFA, 6 ml) at 110 °C for 3 h. After TFA was removed by vacuum evaporation, the mixture was divided into two parts. One part of the hydrolyzate was analyzed with thin layer chromatography (TLC) on a plate (10 × 20 cm) pre-coated with the slurry made of silica gel (6 g), $NaH_2PO_4{\cdot}2H_2O$ (0.75 g) and 0.5% CMC-Na (16 ml). The developing agent was a mixture of acetic ether, pyridine, water, and acetic acid (5:5:3:1). The sugars were identified by spraying *o*-phthalic acid reagent (1.6 g of *o*-phthalic acid dissolved in 100 ml of water-saturated *n*-butanol, containing 0.9 ml of aniline) onto the plate and heating at 105 °C for 5 min. The other part was reduced with $NaBH_4$ and acetylated with acetic anhydride-pyridine (Blakeney, Harris, Henry, & Stone, 1983). The resulting alditol acetates were analyzed by gas–liquid chromatography (GLC) using a Agilent HP6890 instrument equipped with a DB-5 capillary column (30 m × 0.25 mm) and a flame-ionization detector. The temperature program was from 120 to 250 °C with a rate of 6 °C/min and at 250 °C for 10 min. Peaks were identified and estimated with *myo*-inositol as the internal standard. Quantitation was carried out from the peak area, using response factors.

2.6. Methylation analysis

PRP was methylated four times by the Needs and Selvendran's method (1993). Complete methylation was confirmed by the lack of hydroxyl peak in IR spectrum. The permethylated product was depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 110 °C for 3 h. The residues were reduced and acetylated. The resulting products were analyzed by GLC-MS. The GLC temperature program was isothermal at 120 °C, followed by a 6 °C /min gradient up to 250 °C and at 250 °C for 10 min. The partially methylated alditol acetates were identified by their fragment ions in GLC-MS and by relative retention time on GLC, and the molar ratios were estimated from the peak areas and the response factors (Björndal, Lindberg, & Svensson, 1967).

2.7. Periodate oxidation and Smith degradation

PRP (20 mg) was dissolved in 0.015 M $NaIO_4$ (30 ml) and kept in dark at 4 °C. The $NaIO_4$ consumption was monitored by a spectrophotometric method each day

(Aspinall & Ferrier, 1957; Linker, Evans, & Impallomeni, 2001). After complete oxidation identified with a stable absorbance achieved (144 h), ethylene glycol (0.2 ml) was added to decompose the remaining $NaIO_4$. Formic acid production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ and dialyzed again. The nondialysate was lyophilized, and then hydrolyzed with 2 M TFA at 110 °C for 2 h. The hydrolysate was converted into its respective alditol acetates as above and analyzed by GLC.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

PRP (30 mg) was dissolved in D_2O (0.5 ml). The ^{13}C NMR, ^{13}C DEPT (distortionless enhancement by polarization transfer) and ^{1}H NMR spectra were recorded at 40 °C with a Bruker Avance 600 MHz spectrometer (Germany). The chemical shifts were expressed in ppm relative to the resonance of internal standard DSS. The DEPT experiment was carried out using a polarization transfer pulse of 135. Two-dimensional NMR techniques were used for general assignments of PRP: $^{1}H,^{1}H$ COSY ($^{1}H,^{1}H$ correlation spectroscopy), $^{1}H,^{13}C$ HMQC ($^{1}H,^{13}C$ heteronuclear multiple quantum coherence).

2.9. Mild acid hydrolysis

PRP (100 mg) was dissolved in 0.2 M TFA (10 ml), hydrolyzed at 100 °C for 4 h, and then neutralized to pH 7.0 with 0.5 M NaOH. The hydrolysate was dialyzed against distilled water, and the nondialysate was lyophilized to give PRP-1 for ^{13}C NMR spectrum.

2.10. Spleen cell proliferation assay

The spleen of a Kunming mouse (20 ± 2 g) was removed, minced and passed through a sterilized ion mesh (100 mesh) to obtain single spleen cell suspension. Erythrocytes in the cell mixture were destroyed by addition of Tris–(hydroxymethyl)-aminomethane (Tris)–NH_4Cl. The spleen cells were washed twice with Hank's solution and suspended to a final density of 5×10^6 cells/ml in RPMI-1640 medium. The above cell suspension was seeded in a 96-well plate with 100 μl per well, then 100 μl of PRP (concentration range: 10–200 μg/ml) and concanavalin A (ConA, 10 μg/ml) were added. After incubation for 48 h at 37 °C in a humidified 5% CO_2 incubator, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT: 5 mg/ml; Sigma, USA) was added into each well. The plate was incubated for another 4 h, 100 μl of 10% SDS in 0.01 M HCl was added to each well to dissolve the colored material for 12 h. The absorbance at 570 nm was measured in an ELISA reader (Mossmann, 1983; Zhang, Shen, Li, & Sun, 2001).

3. Results and discussion

The yield of the crude water-soluble polysaccharide from *P. ribis* was 2.77% of the dry material. The yied of TPRP was 46.51% of the crude polysaccharide. The TPRP was separated and sequentially purified through DEAE-cellulose and Superdex 30 column chromatography and a purified *P. ribis* polysaccharide (PRP) was obtained. The total sugar content of PRP was 97.3%. As determined by *m*-hydroxydiphenyl colorimetric method, the polysaccharide did not contain uronic acid.

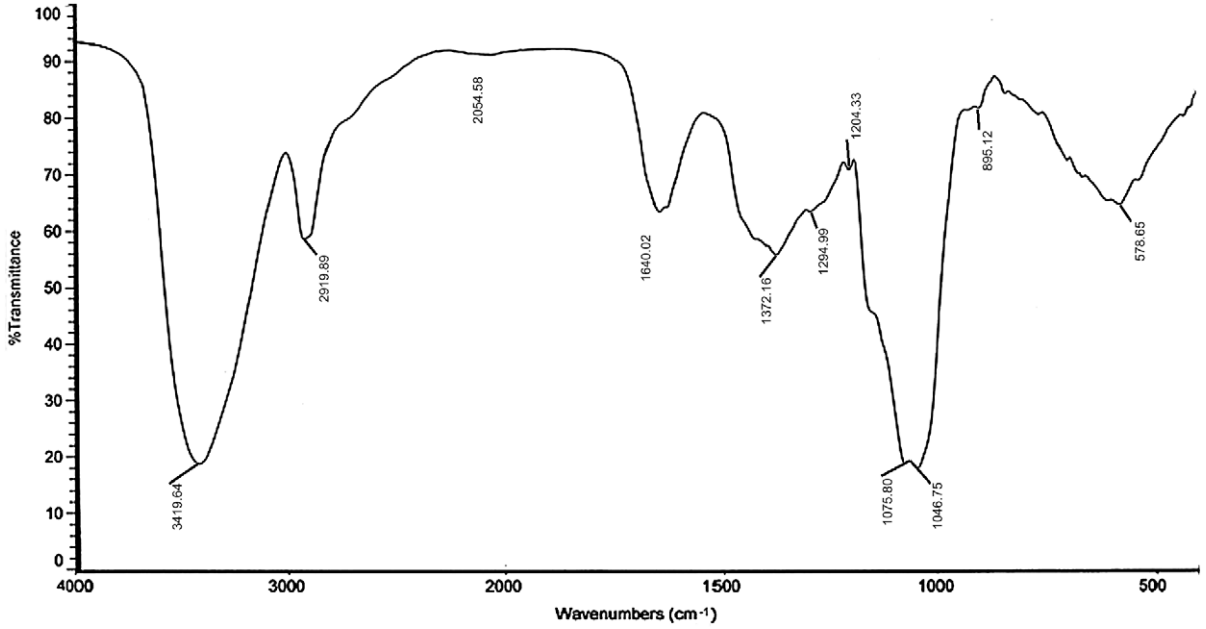

Fig. 1. FT-IR spectrum of PRP.

PRP appeared as a white powder with $[\alpha]_D^{20}$+16.8° (*c* 0.1, H_2O). It had a negative response to the Lowry test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. The HPGPC profile showed a single and symmetric peak, indicating it is a homogeneous polysaccharide. Correlation with the calibration curve of dextran standards, the weight-average molecular weight of PRP was about 8.59 kDa. PRP was composed of only glucose, as detected by TLC of the PRP hydrolyzate and GLC of the alditol acetate derivatives of the PRP hydrolyzate.

The strong absorption in the range of 1200–1000 cm^{-1} that appeared in IR spectrum (Fig. 1) suggested that the monosaccharide in PRP had a pyranose ring. The broad band at 3419.64 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The band at 2919.99 cm^{-1} was due to C–H stretching vibration and the band at 1640.02 cm^{-1} was due to the bound water (Park, 1971). A characteristic absorption at 895.12 cm^{-1} was also observed, indicating the β-configuration of the sugar units, which is in agreement with the data of the low positive specific rotation (Bao, Liu, Fang, & Li, 2001).

Periodate oxidation of PRP resulted in 1.43 mol periodate consumed and 0.53 mol formic acid produced per sugar residue. After further Smith degradation of the periodate-oxidized PRP, the glycerol, erythritol and glucose were found with an approximate molar ratio of 5.0:3.0:1 by GLC after conversion to the corresponding alditol acetates. It was thus deduced that the molar ratio of (1 → 6)-linked (terminal), (1 → 4)-linked and (1 → 3)-linked glucosyl residues is 5.0:3.0:1. Methylation analysis by CLC-MS revealed that PRP consists of (1 → 6)-linked, (1 → 4)-linked, (1 → 3, 6)-linked and terminal glucosyl residues in a molar ratio of 4:3:1:1 (Table 1), in accordance with the

Table 1
GLC-MS data of alditol acetate derivatives from the methylated product of PRP

Methylated sugars (as alditol acetates)[a]	Type of linkage	Retention time[b]	Molar ratio	Mass fragments (*m*/*z*)
2,3,4,6-Me4-Glc	Terminal Glc*p*	1.00	1.00	43,71,87,101,117,129,145,161,205
2,3,6-Me3-Glc	1,4-Linked Glc*p*	1.10	2.85	45,87,99,101,113,117,131,173,233
2,3,4-Me3-Glc	1,6-Linked Glc*p*	1.16	3.71	71,87,99,101,117,129,159,161,173,189,233
2,4-Me2-Glc	1,3,6-Linked Glc*p*	1.24	1.04	87,101,117,129,139,159,189,201,231,233

[a] 2,3,4,6-Me4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose, etc.
[b] Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

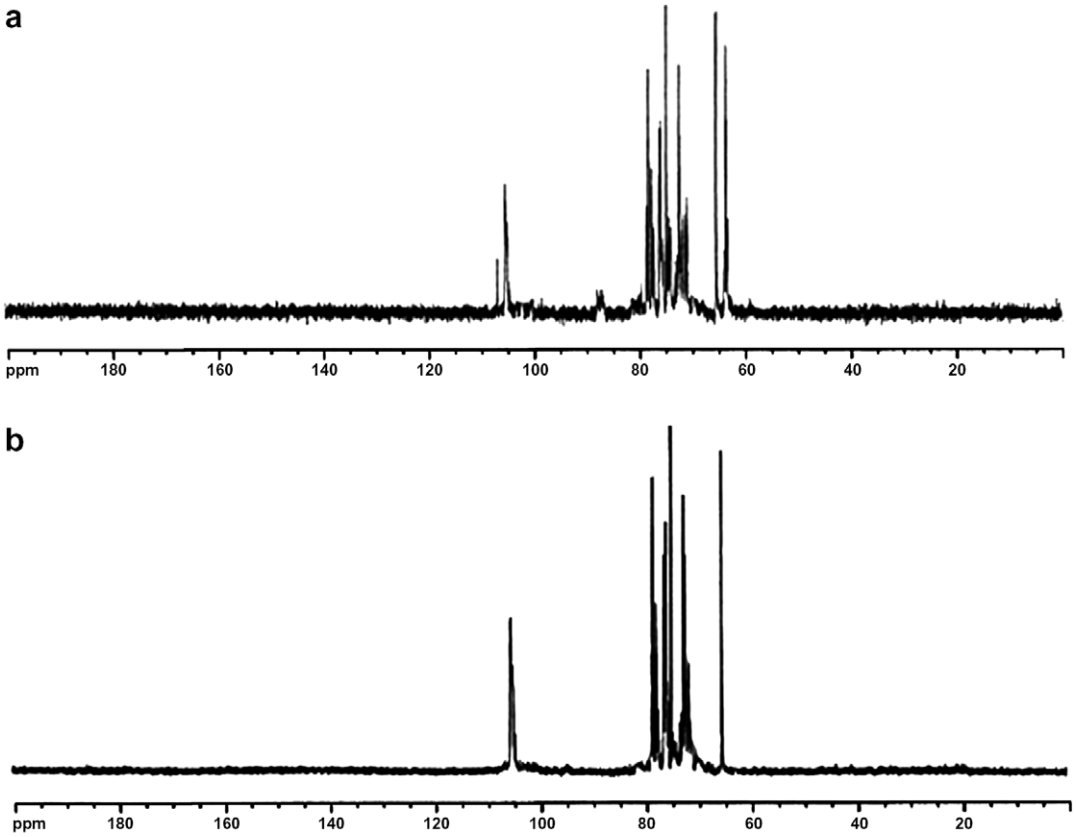

Fig. 2. ^{13}C NMR (600 M) spectrum of PRP (a) and PRP-1 (b).

mode of linkage of glucose present in the polysaccharide by periodate oxidation and Smith degradation. The results suggested that $(1 \rightarrow 6)$-linked glucosyl residues reside in the backbone of PRP, but the position of $(1 \rightarrow 4)$-linked glucosyl residues was uncertain. Therefore mild hydrolysis was performed to give PRP-1 and ^{13}C NMR spectra of PRP and PRP-1 were recorded to determine the structural features of PRP.

Compared with signals in ^{13}C NMR spectrum of PRP, C-1 signal at δ 105.61 and C-6 signal at δ 65.20 of $(1 \rightarrow 4)$-linked glucosyl residues and signals of $(1 \rightarrow 6)$-linked glucosyl residues were found in ^{13}C NMR spectrum

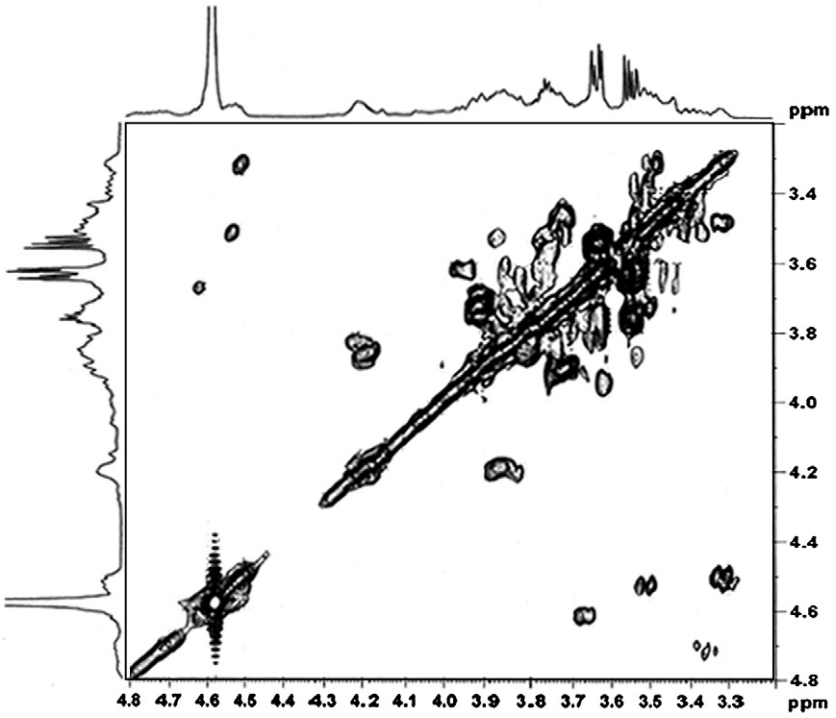

Fig. 3. $^{1}H,^{1}H$ COSY (600 M) spectrum of PRP.

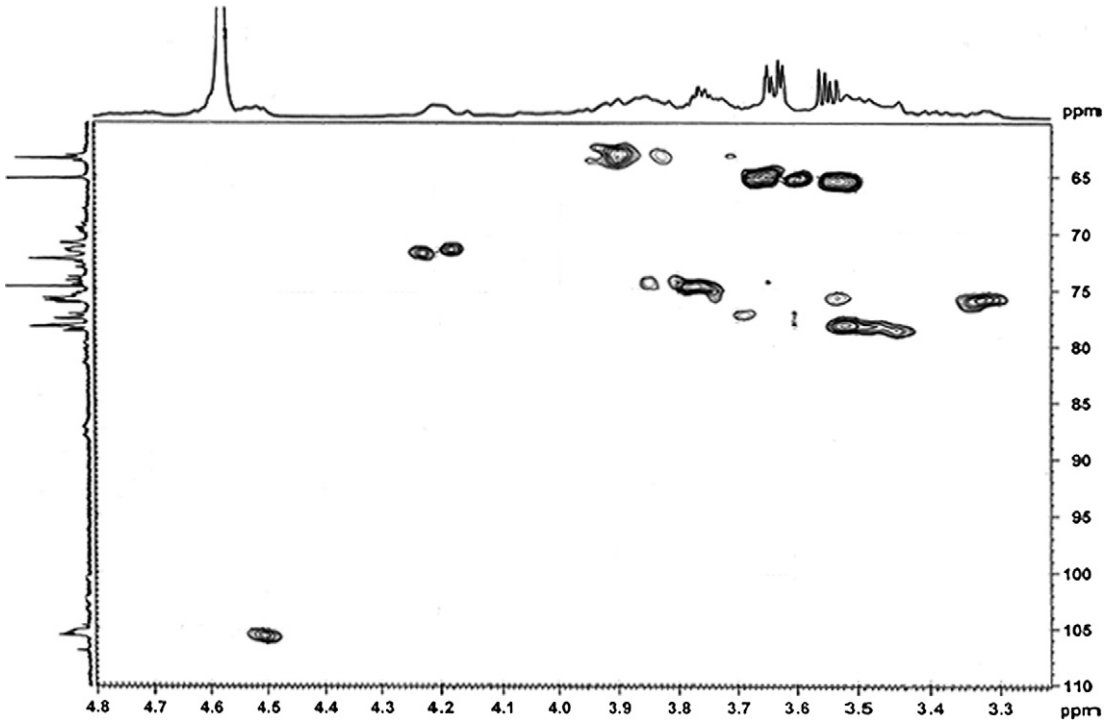

Fig. 4. $^{1}H,^{13}C$ HMQC (600 M) spectrum of PRP.

of PRP-1 (Fig. 2), but C-1 signal at δ 106.99 and C-6 signal at δ 63.41 arising from terminal glucosyl residues disappeared, indicating PRP has a possible $(1 \rightarrow 4)$, $(1 \rightarrow 6)$-linked backbone, and the terminal glucosyl residues reside in side chains of PRP. The assignment of signals above was confirmed by the following DEPT, $^{1}H,^{1}H$ COSY and $^{1}H,^{13}C$ HMQC spectra.

The ^{13}C NMR spectrum (Fig. 2) contained three anomeric signals at δ 106.99, 105.61 and 105.27, assigned to C-1 of terminal, $(1 \rightarrow 4)$-linked and $(1 \rightarrow 6)$-linked glucosyl residues, respectively, further indicating a β-anomeric configuration for glucopyranosyl units (Hall & Johnson, 1969). The signal at δ 71.41 was assigned to *O*-substituted C-6 of $(1 \rightarrow 6)$-β-D-glucosyl residues and δ 63.41 and 65.20 should be unsubstituted C-6 of terminal and $(1 \rightarrow 4)$-β-D-glucosyl residues, which was confirmed from the inverted signals of the DEPT spectrum (not shown). The C-3 signal of $(1 \rightarrow 3,6)$-β-D-glucosyl residues showed a downfield shift due to the glycosylation effect and appeared at 87.15 ppm.

The anomeric signals of PRP were difficult to identify in ^{1}H NMR (not shown), while the correlation of H-1and H-2 appeared in $^{1}H,^{1}H$ COSY (Fig. 3). From the COSY spectrum, three groups of correlated signals were observed: H-1/H-2 at δ 4.52/3.31, H-2/H-3 at δ 3.31/3.49, H-3/H-4 at δ 3.49/3.43; H-1/H-2 at δ 4.53/3.51, H-2/H-3 at δ 3.51/3.87, H-3/H-4 at δ 3.87/3.72, H-4/H-5 at δ 3.72/3.85, H-5/H-6a,H-6b at δ 3.85/4.18, 4.25; H-1/H-2 at δ 4.62/3.68, H-2/H-3 at δ 3.68/3.78, H-3/H-4 at δ 3.78/3.69, H-4/H-5 at δ 3.69/3.77, H-5/H-6a,H-6b at δ 3.77/3.82,3.90. The $^{1}H,^{13}C$ HMQC (Fig. 4) spectrum of PRP revealed information on correlation of ^{13}C and its linked ^{1}H: C-1/H-1 at δ 105.61/4.52, C-6/H-6a,H-6b at δ 65.20/3.55,3.64 for $(1 \rightarrow 4)$-β-D-glucosyl residues; C-1/H-1 at δ 105.27/4.53, C-6/H-6a,H-6b at δ 71.41/4.18, 4.25 for $(1 \rightarrow 6)$-β-D-glucosyl residues and others. Based on the data available in the literature and combination with the above 2D $^{1}H,^{1}H$ COSY and $^{1}H,^{13}C$ HMQC spectra, most of the signals of ^{1}H NMR and ^{13}C NMR were assigned in Table 2.

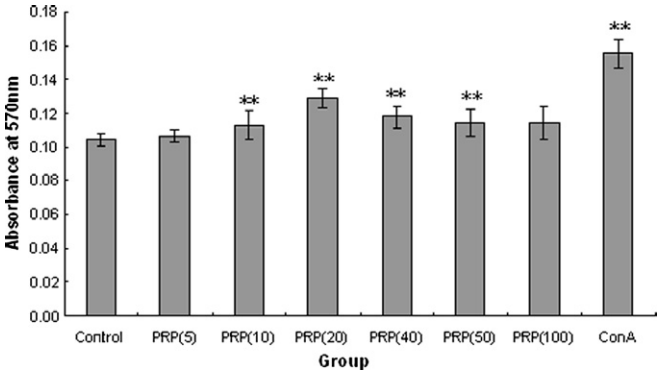

Fig. 6. Effect of PRP (μg/ml) on splenocyte proliferation. Proliferation activity was expressed as the absorption at 570 nm. Values are means ± SD of six, $^{**}P < 0.01$ *vs.* control group.

On the basis of the above-mentioned results, it can be concluded that PRP is a β-D-glucan, which has a $(1 \rightarrow 4)$, $(1 \rightarrow 6)$-linked backbone, with branches at the C-3 position of $(1 \rightarrow 6)$-linked glucosyl residues. The predicted structure is shown in Fig. 5, it is possible that the terminal glucosyl residue is linked to another $(1 \rightarrow 6)$-linked glucosyl residue.

Spleen cell proliferation assay showed that PRP could stimulate the proliferation of spleen lymphocyte, especially at a dose of 20 μg/ml (Fig. 6), suggesting a strong immunostimulatory activity.

4. Conclusion

The polysaccharide PRP, isolated from the fruiting bodies of *P. ribis* for the first time, is a β-D-glucan containing $(1 \rightarrow 4)$, $(1 \rightarrow 6)$-linked backbone, branched $(1 \rightarrow 3)$-linkage. Preliminary activity tests *in vitro* revealed that PRP could stimulate the proliferation of spleen lymphocyte.

Table 2
Partial ^{1}H and ^{13}C NMR chemical shifts of polysaccharide PRP in D_2O

Sugar residue	C/H (ppm)[c]					
	1	2	3	4	5	6
→4)-β-D-Glc*p*-(1→	105.61/4.52	75.82/3.31	78.30/3.49	78.57/3.43	74.71/3.75	65.20/3.64[a], 3.55[b]
→6)-β-D-Glc*p*-(1→	105.27/4.53	76.00/3.51	77.56/3.87	72.26/3.72	74.19/3.85	71.41/4.25[a], 4.18[b]
β-D-Glc*p*-(1→	106.99/4.62	74.14/3.68	78.23/3.78	70.86/3.69	74.71/3.77	63.41/3.90[a], 3.82[b]

[a] Interchangeable.
[b] Interchangeable.
[c] In ppm downfield relative to the signal for DSS.

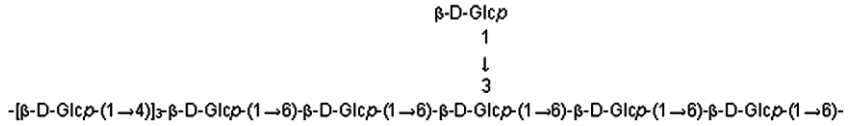

Fig. 5. Possible structure of PRP.

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