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Structure identification of a new immunostimulating polysaccharide from the stems of *Dendrobium huoshanense*

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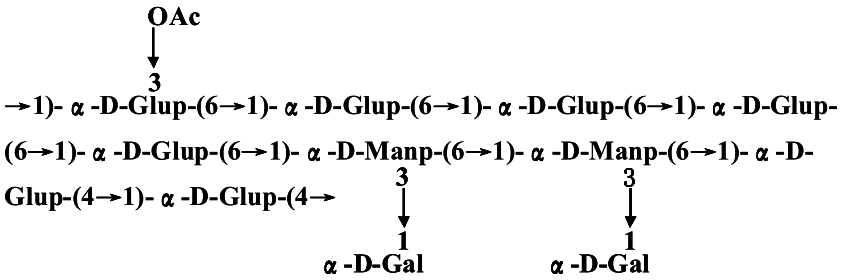
Abstract

The water-soluble polysaccharide HPS, obtained from the stems of *Dendrobium huoshanense* by hot-water (50–60 °C) extraction and ethanol precipitation, was fractionated by DEAE–Cellulose anion-exchange and gel filtration chromatography including Sephacryl S-200 and Sephadex G-75/G-100, giving one polysaccharide fraction of HPS-1B23 which was investigated by chemical techniques and NMR spectroscopy. This polysaccharide fraction consists of glucose, mannose and galactose in the ratio of 31:10:8. On the basis of periodate oxidation–smith degradation, methylation analysis and partial acid hydrolysis, the repeating unit of HPS-1B23 was established:



Pharmaceutical experiments showed that the polysaccharide of HPS-1B23 possessed potent stimulating functions on IFN-γ and TNF-α production in the culture medium of splenocytes and macrophages, respectively.

Keywords: *Dendrobium huoshanense*; Polysaccharide; Structure; Splenocytes; Macrophages; Cytokine

1. Introduction

Herba *Dendrobii* (Shi-Hu) is prepared from the stems of several orchid species belonging to genus *Dendrobium* and has been used in traditional Chinese medicine as a therapeutic agent for curing cataract, throat inflammation, fever and chronic superficial gastritis or as a tonic for promoting the production of body fluid and improving the quality of life (Bao, Shun, & Chen, 2001). Research focused on its constituents and pharmacological activity has shown that there are several active constituents, such as alkaloids, stilbenoids, glycosides and polysaccharides (Chen & Guo, 2000). Polysaccharides have been isolated from some *Dendrobium* species, and most of them are composed of β-(1 → 4)(1 → 6)-linked-mannose, β-(1 → 3)-linked-mannose, β-(1 → 4) -linked-mannose or glucose (Hua, Zhang, Fu, Chen, & Chan, 2004; Wang, Zheng, & He, 1988; Zhao, 1994). However, up to now, studies on polysaccharides from *D. huoshanense* have not been reported. Previous results also showed that polysaccharides having branches

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for (1 → 3)-linked or (1 → 6)-linked glycosyl residues are often reported to be pharmacologically active (Deters, Dauer, Schnetz, Fartasch, & Hensel, 2001; Demleitner, Kaus, & Franz, 1992). In the present study, we report the isolation, purification, properties, structural features and immunostimulatory activity of a new polysaccharide.

2. Materials and methods

2.1. Plant materials and source of sample

Dendrobium huoshanense was collected in Anhui province of China in July, 2004 and propagated under controlled conditions (Luo, Zha, & Jiang, 2003; Zha, Luo, Shi, & Jiang, 2006). The stems were crushed into a powder after being dried in an over. Sephacryl S-200, DEAE–Cellulose and Sephadex G-25/G-75/G-100 were purchased from Amersham Pharmacia Co. and Sigama Co., respectively. Dextrans were from Fluka. All reagents used in this paper were analytical grade.

2.2. General methods

Evaporation was performed at around 40 °C under reduced pressure, and the products were dried by lyophilization. Gas chromatography–mass spectrometry (GC–MS)[1] were used for identification and quantification. Analysis by GC–MS was done on a Trace GC2000/Trace MS chromatography fitted with a fused silica capillary column (0.25 μm × 0.25 mm × 30 m) of HP-5. The column oven was first cooled to 50 °C for 3 min, and then the temperature was raised to 250 °C at 10 °C/min and maintained for 20 min. GC was performed on a Shimadzu GC-9A instrument equipped with a hydrogen flame ionization detector, using a 3% OV-225 column (0.25 mm × 28 m i.d.) at a temperature program of 170 °C (1 min) followed by 1 °C/min to 180 °C (1 min) and 4 °C/min to 250 °C. The hydrogen flow rate was 20 ml/min and the ion-source temperature was 150 °C. Gel filtration chromatography was carried out on columns of Sephacryl S-200, Sephadex G-75 and Sephadex G-100 using 0.2 M NaCl. All gel filtration chromatography was monitored with phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Isolation and purification of polysaccharides

The powdered material (20 g) was pre-extracted for 48 h in a Soxhlet system with acetone and subsequently for another 48 h with methanol. The extract were discarded and the residue was dried at 40 °C for 24 h. Subsequently, the residue was extracted three times with 100 ml hot distilled water (50–60 °C). The whole extract was filtered and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was concentrated to 60 ml and precipitated by the addition of ethanol in 1:4 ratio (v/v) at room temperature (RT). After overnight precipitation, the sample was centrifuged as described above, and the precipitation was dissolved in 60 ml of distilled water. This process was repeated three times. The precipitate was then washed with sevage reagent (isoamyl alcohol and chloroform in 1:4 ratio) (Staub, 1965) and freeze dried, giving the crude polysaccharide *D. huoshanense* (HPS).

Dissolved HPS (2.0% w/v) was fractionated on DEAE–Cellulose anion-exchange column (1.6 cm × 60 cm; NH_2^-, first with water, and then with a gradient (0.1–0.6 M NaCl). HPS-1 was obtained by water elution. HPS-1B was separated from HPS-1 by gel-permeation chromatographic on a Sephacryl S-200 column (1.6 cm × 60 cm), and then HPS-1B2 was purified via a Sephadex G-75 column (1.6 cm × 60 cm). HPS-1B2 further purified on Sephadex G-100 (1.6 cm × 60 cm) and HPS-1B23 was obtained.

2.4. Homogeneity and molecular weight

Samples were detected and determined with high-performance liquid chromatography (HPLC) system, equipped with Ultrahydrogel™ linear column (7.8 × 300 mm, Part No. WAT011545) and Ultrahydrogel™ 500 column (7.8 × 300 mm, Part No. WAT11530) connected in series. The temperature of the column was kept at 34 °C and the sensitivity of the waters-2410 refractive index detector was four. The linear regression was calibrated with Dextrans 31418, 31420, 31422, 31424 and 31425. The sample concentration was 1% (w/v), and its injection volume was 20 μl. The eluent was KNO_3 (0.005 M), at a flow rate of 0.5 ml/min.

2.5. Monosaccharide analysis

HPS-1B23 (10 mg), dissolved in 2 M trifluoroacetic acid (TFA, 2 ml), was hydrolyzed at 120 °C for 3 h. The hydrolyzate was repeatedly co-concentrated with methanol, followed by successive reduction with $NaBH_4$ and acetylation with acetic anhydride–pyridine (1:1, v/v; 2 ml) at RT for 12 h. The resulting alditol acetates were analyzed by GC–MS and GC (conditions as in Section 2.2).

2.6. Periodate oxidation–smith degradation

For analytical purpose, 25 mg of the polysaccharide (HPS-1B23) was dissolved in 12.5 ml of distilled water and 12.5 ml of 30 mM $NaIO_4$ were added. The solution was kept in the dark at RT, 0.1 ml aliquots were withdrawn at 3–6 h intervals, diluted to 25 ml with distilled water and read in a spectrophometer at 223 nm (Deters et al., 2001). Ethylene glycol (2 ml) was added at the end of the reaction which continued for 3 days. The solution of the periodate product (2 ml) was sampled to calculated the yield of formic acid by 0.005 M sodium hydroxide, and the rest was extensively dialyzed against tap water and then distilled

[1] *Abbreviation used:* GC–MS, Gas chromatography–mass spectrometry; GC, gas chromatography; RT, room temperature; HPLC, high-performance liquid chromatography, HBSS, Hank's balanced salt solution.

water for 24 h in each avenue. The content inside was concentrated and reduced with sodium borohydride (80 mg), and the mixture was left for 24 h at RT, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, then lyophilized and hydrolyzed with 2.0 M TFA at 100 °C for 6 h. The hydrolyzate was finally converted into alditol acetates which were analyzed by GC–MS and GC (procedure and conditions described in Sections 2.2 and 2.5).

2.7. Methylation analysis

Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide according to the modified Ciucanu method as described by Needs & Swlvendran (1993). The methylated products was hydrolyzed with 2 M TFA for 3 h at 120 °C and converted into partially methylated aditol acetates. The resulting products were analyzed by GC–MS and GC. Temperature programme of GC was carried out as described previously (Bao, Liu, Fang, & Li, 2001). Partially methylated aditol acetates were identified by their fragment ions in EI-MS and by relative retention times in GC, and the molar ratios were estimated from the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975).

2.8. Partial hydrolysis with acid

Polysaccharide sample was hydrolyzed with 0.05 M TFA, kept at 95 °C for 16 h, centrifuged, dialyzed the supernatant with distilled water for 48 h, and then diluted the solution in the dialysis tube with ethanol. After hydrolysis, the precipitate and supernatant in the dialysis tube and the fraction out of dialysis tube were dried, and then GC analysis was carried out as described in the Sections 2.2 and 2.5.

2.9. NMR spectroscopy

HPS-1B23 (20 mg) was deuterium exchanged several times by lyophilising from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and δ 31.07 for ^{13}C). NMR spectra were recorded at 50 °C on a Bruker Avance AV400. Data processing was performed using standard Bruker XWIN-NMR software.

2.10. Measurement of immunomodulating activity

Animals and preparation of spleen cells and peritoneal macrophages: Male BALB/c mice (18 ± 2 g, 8–10 weeks old) were purchased from the Experimental Animal Center, Anhui Medical University of China. After mice were sacrificed, peritoneal macrophages were recovered by washing the peritoneal cavity with ice Hank's balanced salt solution (HBSS) and the murine splenocytes were obtained by homogenizing spleen with ice cold HBSS. For splenocytes preparation, the single cell suspension from the teased murine was obtained by passing it through a 200-mesh and hemolysed by the buffer solution containing 1 mmol l^{-1} Tris–HCl and 1% NH_4Cl (pH 7.2). Cells were washed twice with RPMI 1640 medium and subsequently suspended in complete RPMI 1640 culture medium. Cell viability was determined by trypan blue dye exclusion and the cell concentration was adjusted to 2.0×10^6/ ml.

Cytokine bioassay. For interferon γ (IFN-γ) determination, splenocytes (2×10^6 cells/ml) were treated by different concentration of HPS in the presence of ConA (5 μg/ml). After incubation for 6, 12, 24, 48 and 72 h at 37 °C in a humidified 5% CO_2 incubator, the cell supernatants were collected and IFN-γ were measured by ELISA kits according to the recommendation of the manufacturer. As described above where only ConA was replaced by LPS (10 μg/ml), the supernatant of peritoneal macrophages were also collected for analysis of the tumor necrosis factor alpha (TNF-α) by ELISA kits.

3. Results and discussion

Powdered stems of *D. huoshanense* (20 g) was pre-extracted for 48 h in a Soxhlet system with acetone (200 ml) and subsequently for another 48 h with methanol (200 ml) to inactivate the systems and remove pigments and low-molecular-weight substances. After drying at 40 °C for 24 h, the residue was extracted with hot distilled water (100 × 3 ml, 50–60 °C) and the exhaustive extraction gave the crude polysaccharide of *D. huoshanense* (HPS) (18.1%) and subsequent removal of proteins by the sevage method and separation by anion-exchange chromatography on DEAE–Cellulose NH_2^-, gave HPS-1 (81%) from the distilled water eluate (200 ml); and HPS-2 (12%), HPS-3 (5%), HPS-4 (0.9%), HPS-5 (1.1%) were obtained in turn from an aqueous NaCl gradient (0.1, 0.2, 0.3 and 0.6 M). The last three components were in trace amount (Figs. 1A and B). HPS-1 (0.5 g) was separated on a Sephacryl S-200 column, giving two fractions: HPS-1A (10%) and HPS-1B (78%). HPS-1B was further purified on Sephadex G-75 and Sephadex G-100, giving HPS-1B23 which presented a symmetrical and narrow peak on HPLC as shown in Fig. 2. Its average molar weight was estimated to be 2.2×10^4 Da by HPLC, using dextrans of known molecular weight as standards. The optical rotation was $[\alpha]_D^{20} + 130.7$. The polysaccharide was free of proteins as determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Complete hydrolysis of HPS-1B23 with 2 M TFA and examination by GC–MS and GC showed a large proportion of glucose and mannose, with a trace of galactose. Results showed that glucose, mannose and galactose in the molar ration of 31:10:8. The three monosaccharides were assigned the α-pyranose form from anomeric signals in the ^1H and ^{13}C NMR spectra; ^{13}C NMR signals at δ 82–88 ppm characterized as furanoses were absent. Analysis of GC–MS also confirmed that all the monosaccharides were in the D configuration.

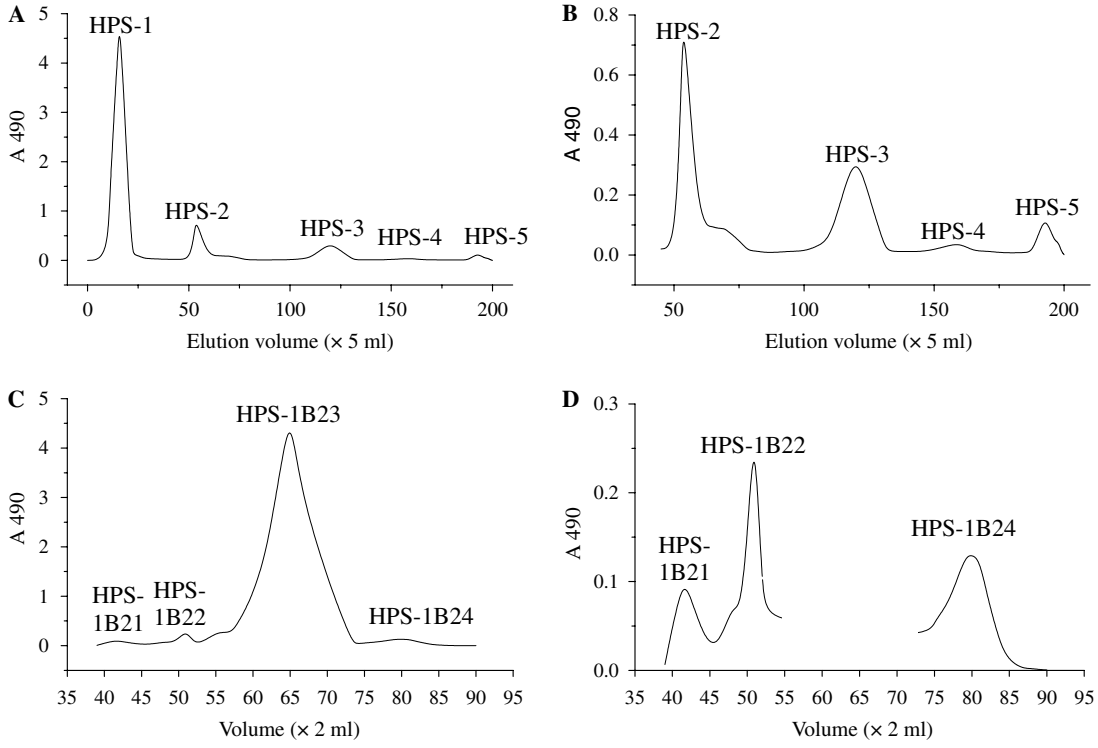

Fig. 1. Chromatogram of HPS. (A) the DEAE–Cellulose column chromatogram of HPS by NaCl gradient elution. (B) an enlargement of DEAE–Cellulose column chromatogram in the 230–1000 ml range. (C) Sephadex G-100 gel-permeation chromatogram. (D) the enlargement of Sephadex G-100 gel-permeation chromatogram in 78–110 and 144–180 ml range.

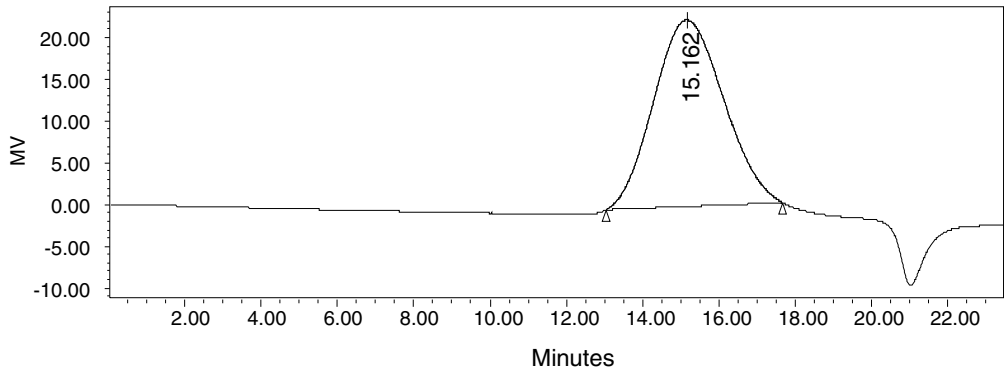

Fig. 2. High-performance liquid chromatogram of HPS-1B23 from *D. huoshanense*.

HPS-1B23 was oxidized with 0.015 M sodium metaperiodate ($NaIO_4$) at RT in the dark for 3 days. A total of 1.25 mol $NaIO_4$ was consumed per mole of sugar residues, based on the average molar mass (160) of a glycosyl residue. The production of formic acid was 0.52 mol. It was thus deduced that the nonreducing terminal residues or $(1 \rightarrow 6)$-linked glycosyl bonds amounted to 52%, with $(1 \rightarrow 2)$-/$(1 \rightarrow 4)$-linked and $(1 \rightarrow 3)$-linked glycosyl bonds amounting to 21% and 27%, respectively. The oxidized products were reduced and hydrolyzed. GC – MS and GC analysis identified the presence of erythritol, glucose, mannose and glycerol in the ratio of 3.2:1.5:1:6.5. The presence of glucose and mannose indicating a part of glucose and mannose are $(1 \rightarrow 3)$-linked, $(1 \rightarrow 2,3)$-linked, $(1 \rightarrow 2,4)$-linked, $(1 \rightarrow 3,4)$-linked, $(1 \rightarrow 3,6)$-linked or $(1 \rightarrow 2,3,4)$-linked, namely linkages that cannot be oxidized. Galactose were absent, then it should be inferred that galactose were all linkages that can be oxidized, namely 1-linked, $(1 \rightarrow 6)$-linked, $(1 \rightarrow 2)$-linked, $(1 \rightarrow 2,6)$-linked, $(1 \rightarrow 4)$-linked or $(1 \rightarrow 4,6)$-linked. The products of glycerol and erythritol suggested the existence of $(1 \rightarrow 6)$/ $(1 \rightarrow 2)$-linked glycosyl residues and $(1 \rightarrow 4)$-linked glycosyl in HPS-1B23, respectively.

Results of methylation linkage analysis of fraction HPS-1B23 are summarized in Table 1. Results showed the presence of four components, namely 2,3,4-Me_3-Glu, 2,3,4,6-Me_4-Gal, 2,4-Me_2-Man and 2,3,6-Me_3-Glu in molar ratio of 2.4:0.9:1:1.1. This showed a good correlation

Table 1
Methylation analysis data for HPS-1B23 from *Dendrobium huoshanense*

Methylated sugar	Mass fragments (*m*/*z*)	Molar ratio	Mode of linkage
2,3,4,6-Me_4-Gal*p*	43, 71, 87, 101, 117, 129, 145, 161, 205	0.9	Terminal
2,3,4-Me_3-Glu*p*	43, 58, 71, 87, 99, 101, 117, 129, 161, 189, 233	2.4	→ 6Glup(→1
2,4-Me_2-Man*p*	41, 43, 58, 71, 87, 99, 117, 129, 159, 173, 189, 233	1.0	→ 3,6)Manp(→1
2,3,6-Me_3-Glu*p*	43, 58, 71, 87, 101, 117, 127, 143, 161, 173, 233	1.1	→ 4)Glup→1

between terminal and branched residues. The terminal galactose groups were attached to (1 → 3,6)-linked mannose, as the ratio between them was around 1:1. In addition, these molar ratios agree with the overall monosaccharide composition of HPS-1B23 described above. Both results of partial acid hydrolysis and methylation linkage analysis of HPS-1B23 indicated that 2,3, 4-Me_3-Glup, 2,3,6-Me_3-Glup and 2,4-Me_2-Manp were the major components of the backbone structure. Compared with the results of periodate oxidation, (1 → 3)-linked glucosyl residues were all absent. It was proposed that groups such as acetyl groups joined to C-3 position of (1 → 6)/(1 → 4)-linked glucosyl residues, forming an unstable glycosyl residue, of which the periodate oxidation results were the same as (1 → 3)-linked glucosyl residues. Furthermore,

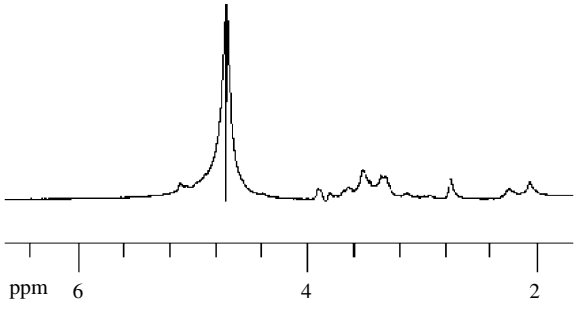

Fig. 3. ^{1}H NMR spectrum of HPS-1B23 from *D. huoshanense* obtained at 50 °C.

the components were converted into (1 → 6)/(1 → 4)-linked glucosyl residues, due to the removal of these unstable groups under the strongly alkaline conditions in the course of repetitive methylation. The product content of erythritol (25.6%) from periodate oxidation was very coincided with that of (1 → 4)-linked glucose (25%) from methylation analysis, therefore, it is logical to deduce that there is not (1 → 3,4)-linked glucose but (1 → 3,6)-linked structure of glucose in HPS-1B23. By analysis of the result of periodate oxidation together with that of methylation, (1 → 6)-linked glucosyl residues, (1 → 4)-linked glucosyl residues, (1 → 3,6)-linked glucosyl residues were found to be in the ratio of 4:2:1.

The NMR sample used for structural studies was viscous and gave relatively broad signals. Assignments of signals and identification of sugar residues were done by combinations of two-dimensional techniques and comparison of the chemical shifts with published data on similarity substituted sugar residues. The anomeric signals in ^{1}H NMR spectrum of HPS-1B23 at δ 5.00–5.20 (Fig. 3) were assigned by comparison with the NMR data reported previously and detailed assignments of all signals are in Table 2 (Vanhaverbeke et al., 1998; Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004; Zhao, Li, Luo, & Wu, 2006; Galbraith, Sharples, & Wilkinson, 1999; Linker, Evans, & Impallomeni, 2001; Kocharova et al., 2001).

The anomeric configuration of each residue in HPS-1B23 was elucidated from ^{13}C NMR spectrum (Fig. 4.). The anomeric region contained five signals at δ 97.8 –

Table 2
^{13}C and ^{1}H NMR chemical shifts (ppm) for the polysaccharide of HPS-1B23 form *Dendrobium huoshanense*

Sugar residues	1	2	3	4	5	6
→ 1α-D-Glu*p*-(6 →						
C	97.8	72.7	73.3	72.1	72.8	68.2
H	5.09	3.53	3.72	3.45	3.92	3.64 (3.87)[a]
→ 1,3)-α-D-Man*p*-(6 →						
C	101.7	71.7	nd[b]	68.1	73.3	69.9
H	5.14	3.94	3.92	3.90	nd	3.75 (3.82)
→ 1)α-D-Glu*p*-(4 →						
C	98.6	72.1	70.5	75.6	69.9	61.8
H	5.16	3.51	3.72	3.52	4.18	3.86 (3.82)
→ 1,)-3-*O*-acetyl-α-D-Glu*p*-(6 →						
C	99.2	71.6	nd	70.5	72.8	67.7
H	5.09	3.63	3.92	3.53	3.90	3.75 (3.85)
→ 1)α-D-Gal*p*						
C	99.0	69.8	70.9	70.3	72.3	61.5
H	5.01	3.72	3.64	nd	3.70	3.72 (3.83)

[a] The value inside and outside the brackets denote the chemical shifts of H-6a and 6b.
[b] nd represents the value cannot be determined.

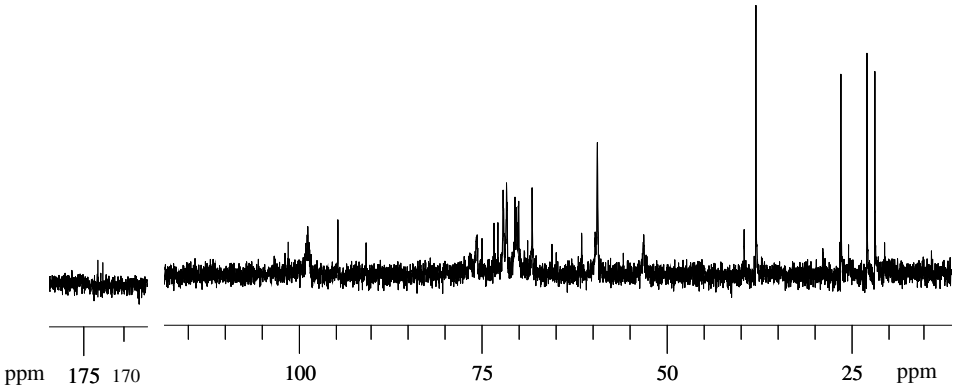

Fig. 4. ^{13}C NMR spectrum of HPS-1B23 from *D. huoshanense* obtained at 50 °C.

101.7. Signals at 97.8 and 98.6 belonged to (1 → 6)-linked and (1 → 4)-linked glucosyl residues, respectively. The signals at δ 99.2 was assigned to C-1 of 3-*O*-acetyl (1 → 6)-linked glucosyl residues, which was shifted unfield (1.4 ppm) significantly as compared with nonacetylated glucose. A signal δ 99.0 was to the terminal residues of (→1)-linked galactose; the remainder represents that of (1 → 3,6)-linked mannose. According to the previous reports, δ 20.3–22.7 and δ 173.6–174.1 were identified to be Me and CO of *O*-acetyl groups. The signals at δ 2.10–2.35 in the ^{1}H NMR spectrum of HPS-1B23 also supported the above deduction. The rest of the observed signals are shown in Table 2 according to the literature data (Vanhaverbeke et al., 1998; Chakraborty et al., 2004; Zhao et al., 2006; Galbraith et al., 1999; Linker et al., 2001; Kocharova et al., 2001).

Four fractions HPSF1, HPSF2, HPSF3 and HPSF4, obtained after partially acid hydrolysis of HPS-1B23, were subjected to GC analysis as alditol acetates (shown in Table 3). Both HPSF1 and HPSF2 contained the monosaccharide glucose and mannose. Further methylation analysis showed that the linkage types were (1 → 4)-linked glucose, (1 → 6)-linked glucose and (1 → 6)-linked mannose. These results suggested that the polysaccharide had a backbone consisting of (1 → 4)-linked glucose, (1 → 6)-linked glucose and (1 → 6)-linked mannose. The fraction of HPSF4 contained large amount of galactose as compared with the molar ratios of sugar composition, indicating that galactose should be present at branch chains. The absence of galactose in HPSF1, HPSF2 and HPSF3 further supported that suggestion. According to partial acid hydrolysis and methylation linkage analysis of HPS-1B23, the terminal residue of (→1)-linked galactose was attached to the C-3 position of (1 → 6)-linked mannose, as the ratio between them was around 1:1 and no other branched residues were observed in methylation analysis. Periodate oxidation, methylation analysis and NMR detection suggested that a part of C-3 position of (1 → 6)-linked glucose were substituted by acetyl groups to the extent of around 20%, namely on average one branching point for each five (1 → 6)-linked glucose of backbone, and also revealed that (1 → 6)-linked glucose, (1 → 4)-linked glucose, (1 → 3,6)-linked and (1 → 3,6)-linked mannose were in the molar ratio of 4:2:1:2.2 in backbone. Therefore, the suggested repeat unit of HPS-1B23 is indicated in Fig. 5.

Table 3
GC analysis results of fractions from partial acid hydrolysis

Fractions	Molar ratios		
	Gal	Man	Glu
HPSF1[a]		0.38	1
HPSF2[b]		0.51	1
HPSF3[c]		0.42	1
HPSF4[d]	1.4		1

[a] Precipitate.
[b] Precipitate in the dialysis tube.
[c] Supernatant in the dialysis tube.
[d] Fraction out of dialysis tube.

Macrophages are the antigen-presenting cells known to be both cytotoxic and phagocytic to invading microorganisms. Stimulation of macrophages enhances these functions and might therefore be a target for therapeutic applications. TNF-α, one of the cytokines released by macrophages, plays a critical role in mediating signal transduction and stimulating the immune defense system (Liu, Li, Kong, Lin, & Yang, 1998). It has been suggested that polysaccharides in some plants can stimulate the immune system (Liu, Ooi, & Fung, 1999; Bao, Wang, Fang, & Li, 2002; Qiu, Jones, Wylie, Jia, & Orndorff, 2000; Wang, Fang, Ge, & Li, 2000; Wong, Leung, Fung, & Cho, 1994; Xiang & Li, 1993). We hypothesized that polysaccharides of *D. huoshanense* might also stimulate macrophage to release TNF-α thereby stimulating the immune system. To test this hypothesis, HPS-1B23 at

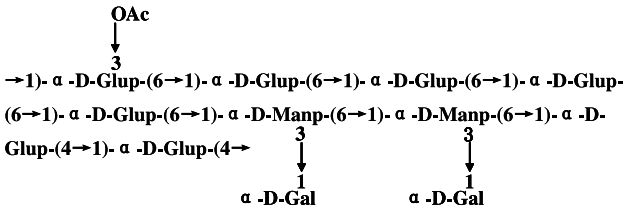

Fig. 5. Repeat unit of HPS-1B23 from *D. huoshanense*.

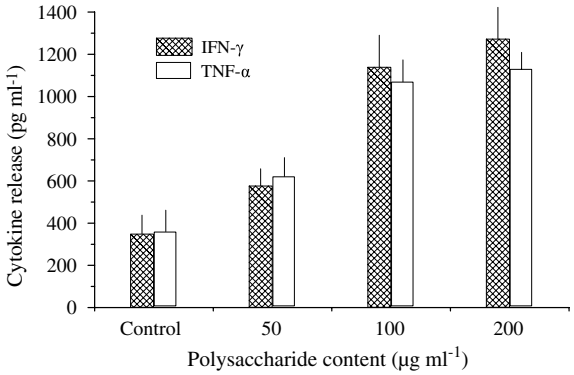

Fig. 6. Effect of HPS-1B23 from *D. huoshanense* on TNF-α and IFN-γ release in culture medium of Macrophages and spleen cells, respectively.

different concentrations was incubated with mouse peritoneal macrophages for 24 h. There was no observed toxicity effect of the samples on cell viability at the experimental concentration range as determined by trypan blue exclusion. The in vitro study showed that HPS-1B23 possessed an enhancing effect on TNF-α production and the peak of 1130.4 pg ml^{-1} was obtained in the culture medium with 200 μg ml^{-1}.

Lymphocytes are also important immune cells and play a pivotal role in immune responses. These cells are able to produce many kinds of cytokines after differentiation and activation. As the first step for T cell activation, lymphocytes should be activated by various signals such as LPS or plant products including polysaccharides (Bao et al., 2002; Cho, Kim, Yoo, Baik, & Park, 2002). The immune response can be broadly categorized into cellular- or humoral-mediated response. The two types of immune responses are separately regulated by cytokines that control two general subsets of helper cells known as Th1 and Th2. IFN-γ is evaluated as representative Th1 cytokines mainly secreted from Th1 cells (Hsieh et al., 1993). Therefore in this study, IFN-γ was determined in splenocytes culture medium and the highest value was also found in that treated with 200 μg ml^{-1} HPS in the presence of 5 μg ml^{-1} ConA by the end of culture, which was 3.6-fold that of the blank experiment (Fig. 6).

In summary, it was concluded that the water extract of *D. huoshanense* contained one predominant water extractable polysaccharide (HPS-1B23). It was α configuration, and its repeating unit of structure was proposed as described above. According to their estimated molecular weight, it was possible assume that HPS-1B23 contained 12 repeating units. Furthermore, this polysaccharide has high immunostimulating activity.

Acknowledgements

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