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Structures of (1 → 6)-β-D-glucans from *Bulgaria inquinans* (Fries) and their immunological activities

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ABSTRACT

In previous study, an unbranched (1 → 6)-β-D-glucan with Mw 2.6 kDa was isolated from fruit bodies of *Bulgaria inquinans* (Fries). In present paper, three branched (1 → 6)-β-D-glucans were obtained from the water-extracted residues by a sequential KOH-extraction, namely BIK2, BIK10 and BIK30. Their molecular weights were determined to be 37.5 kDa (BIK2), 288.9 kDa (BIK10) and 175.5 kDa (BIK30). Structural analysis indicated that their backbones were substituted by single glucosyls at C-3 positions, the branching ratios were 0.01 (BIK2), 0.17 (BIK10), 0.25 (BIK30). Immunological tests showed that all the four β-D-glucans could significantly increase the ConA or LPS-induced lymphocytes proliferation *in vivo*. Moreover, branched (1 → 6)-β-D-glucans have more significantly lymphocytes proliferation activities than unbranched (1 → 6)-β-D-glucan, and the effect of (1 → 6)-β-D-glucans on lymphocytes proliferation increases along with molecular weights. The present results well enrich the structure–activity relationships of (1 → 6)-β-D-glucan, and indicate (1 → 6)-β-D-glucans from *B. inquinans (Fries)* are potential immunostimulating agents.

1. Introduction

Mushrooms have been valued as edible and medicinal resources, in which many biological active substances have been identified (Chatterjee, Biswas, Basu, & Acharya, 2011; Largeteau, Llarena-Hernandez, Regnault-Roger, & Savoie, 2011). Polysaccharides are the best known and most potent mushroom-derived substances with immunomodulating properties (Bisen, Baghel, Sanodiya, Thakur, & Prasad, 2010; Wong, Lai, & Cheung, 2011). Fungal polysaccharides are mainly glucans with various structures (Feofilova, 2010). It is now known that (1 → 3)-β-D-glucans and (1 → 6)-β-D-glucans, including those of mixed β-(1 → 3, 1 → 6) glucosidic linkages, are biological response modifiers (BRMs) as they are able to stimulate the nonspecific immune system of animals (Noss et al., 2012; Rondanelli, Opizzi, & Monteferrario, 2009). Therefore, study on fungal β-glucans is a hot pot in the research field of natural medicine and functional food.

Bulgaria inquinans (Fries) is a non-lichenized edible ascomycete growing on freshly felled oak commonly in the Changbai Mountain area of China, and used as food and folk antitumor medicine for many years (Huang, 1998). Bioassay tests showed that some small molecules from *B. inquinans* (Fries), including benzofluoranthrene derivatives, dihydroxyperylenequinones and azaphiones, had antitumor, antipruritic and antierythema activities (Jiang, Tsumuro, Takubo, Fujii, & Kamei, 2005). In previous study, we isolated and characterized a low molecular weight (1 → 6)-β-D-glucan and a heteropolysaccharide composed of mannose, glucose and galactose from the fruit bodies of *B. inquinans* (Fries) (Bi et al., 2009, 2011). The present paper is concerned with the isolation and structure characterization of three (1 → 6)-β-D-glucans from *B. inquinans* (Fries). The investigated polysaccharides were also evaluated for their immunological activities in *vivo*.

2. Materials and methods

2.1. Materials

Fruit bodies of *B. inquinans* (Fries) were collected in October at Changbai mountain area in Jilin Province, China and identified by Professor Yutang Zhao, School of Life Sciences, Northeast Normal University in Changchun, China. A voucher specimen (No. 20070802) was deposited at the School of Life Sciences, Northeast Normal University.

2.2. Analytical methods

The total carbohydrate content was determined by the phenol–sulfuric acid method, using glucose as the standard. Protein

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content was determined by the Bradford assay, using bovine serum albumin as the standard. Contaminant endotoxin was analyzed by Limulus amebocyte lysate (LAL) assay using an E-TOXATE kit (Sigma, St. Louis, USA) according to the manufacturer's instruction.

Gas chromatography was performed using a Shimadzu GC-14C instrument equipped with a hydrogen flame ionization detector on an Rtx-2330 column (0.32 mm × 15 m i.d., 0.2 μm). High performance liquid chromatography (HPLC) was performed using a Shimadzu 10Avp HPLC system equipped with 10Avp HPLC Pump, SPD-10Avp UV-VIS Detector.

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2B, China). UV–vis absorbance spectra were recorded with a UV-Vis spectro-photometer (Model SP-752, China). Sepharose CL-6B, standard monosaccharides and gel filtration standard dextrans were purchased from Sigma Co. All other chemicals were of analytical grade made in China.

2.3. Extraction and purification

The isolation and purification of the water-soluble polysaccharides (BIW) from fruit bodies of *B. inquinans* (Fries) was carried out as the procedure established in our group previously, and finally a low molecular weight (1 → 6)-β-D-glucan (BIWP2) was obtained (Bi et al., 2009).

The water-extracted residue was dried, and then extracted with 2% aqueous KOH containing traces of $NaBH_4$ at 95 °C for 3 h (×3, material–liquid ratio is 1:10 w/v each). The extract was neutralized with 36% HOAc, added 95% ethanol up to 80% to precipitate the polysaccharides. After collected by centrifugation, the polysaccharide was dried in vacuum. The collected polysaccharide was dissolved in 0.5 M NaOH (15%, w/v) to remove the insoluble substances originating from centrifugation. The supernatant was treated with Sevag reagent (1:4 n-butanol:chloroform, v/v) to remove proteins. After removing the protein and Sevag reagent by centrifugation, the water phase was neutralized with 36% HOAc, and centrifugated to collect the precipitates. The resulting polysaccharide precipitate was suspended in water and dialyzed against tap water and distilled watered in turn, giving rise to fraction BIK2 (2% aqueous KOH extraction). The residue was submitted to a sequential extraction with 10% and 30% aqueous KOH following the above procedure to obtain fractions BIK10 (10% aqueous KOH extraction), BIK30 (30% aqueous KOH extraction). Dialysis was carried out using a tube with Mw cut-off of 3500 Da (for globular protein).

2.4. Determination of homogeneity and molecular mass

The homogeneity and molecular mass were determined by gel filtration chromatography on AKTA FPLC System (GE Healthcare, USA). Sample (5 mg) was dissolved in 0.5 M NaOH (0.5 mL), and the solution was applied to a Sepharose CL-6B column (85 cm × 1.5 cm i.d.), eluting with 0.5 M NaOH at a flow rate of 0.15 mL/min. The gel filtration column was calibrated by standard dextrans (12 kDa, 50 kDa, 150 kDa, 470 kDa and 670 kDa) using linear regression.

2.5. Monosaccharide composition analysis

The monosaccharide analysis was performed as described by Honda et al. (1989). The polysaccharide sample (2 mg) was hydrolyzed with anhydrous methanol (0.5 mL) containing 2 M HCl at 80 °C for 16 h and then with 2 M CF_3COOH (0.5 mL) at 120 °C for 1 h. The hydrolysis-product was derivatized with 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) and 0.3 M NaOH. After neutralization with 0.3 M HCl, the derivatives were analyzed by HPLC on a Shimpack VP-ODS column (150 mm × 4.6 mm i.d.) with a guard column on a Shimadzu HPLC system (LC-10ATvp pump and UV-VIS detector) and monitored by UV absorbance at 245 nm.

2.6. Periodate oxidation and Smith degradation

The periodate oxidation was performed according to the procedure described by Chaplin and Kennedy (1994). Polysaccharide sample (50 mg) was dissolved in 0.015 M $NaIO_4$ (50 mL), and the solution was kept at 4 °C in dark. The $A_{223\,nm}$ of reaction solution was determined every 12 h by spectrophotometer. After the oxidation was completed, the excessive $NaIO_4$ was decomposed with ethylene glycol (0.1 mL). The $NaIO_4$ consumption was calculated according to the decrease of absorbance ($A_{223\,nm}$). The formic acid production was determined by titration with 0.1 M NaOH. The reaction mixture was dialyzed against tap water and distilled water in turn, and then the retentate was reduced with $NaBH_4$ overnight. After neutralization, dialysis and concentration to a small volume, one-third of the retentate was freeze-dried, hydrolyzed with 2 M CF_3COOH (1 mL) at 120 °C for 2 h, reduced by $NaBH_4$, acetylated with pyridine (0.5 mL) and acetic anhydride (0.5 mL) at 90 °C for 1 h, and then analyzed for sugar composition by GC. Others were added to the same volume of 1 M H_2SO_4, kept for 40 h at 25 °C, neutralized with $BaCO_3$, and the filtrated. The filtrate was dialyzed against distilled water, and the content out of sack was freeze-dried for GC analysis; the content inside was added ethanol up to 80%, and after centrifugation, the supernatant and precipitate were also dried out for the GC analysis.

2.7. Methylation analysis

The methylation analysis was carried out according to the method of Needs and Selvendran (1993). In brief, sample (10 mg) was dissolved in DMSO (1.5 mL) and methylated by treatment with NaOH/DMSO suspension (1 mL) and iodomethane (1.0 mL). The reaction mixture was extracted with $CHCl_3$, and then the solvent was removed by vacuum evaporation. Completed methylation was confirmed by the disappearance of the —OH band (3200–3400 cm^{-1}) in the FT-IR spectrum. The per-O-methylated polysaccharide was hydrolyzed subsequently by HCOOH (85%, 0.5 mL) for 4 h at 100 °C and then CF_3COOH acid (2 M, 1 mL) for 6 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced by $NaBH_4$ and acetylated. The resulting alditol acetates were analyzed by GC–MS.

2.8. NMR analysis

^{13}C NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Germany), operating at 150 MHz. Sample (20 mg) was dissolved in (Me_2SO-d_6, 0.5 mL) and centrifuged to remove the excess sample. Test Temperature is 20 °C. All the data were analyzed using standard Bruker software.

2.9. Lymphocyte proliferation activity assay

ICR mice (Grade II, 6–8 weeks old) weighing 18–22 g, half male and half female, were purchased from Pharmacology Experimental Center of Jilin University (Changchun, China) and acclimatized for 1 week prior to use (24 ± 1 °C, with humidity of 50 ± 10%, and a 12/12 h light/dark cycle). All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by School of Life Sciences of Northeast Normal University and were approved by the university committee for animal experiments.

Mice were randomly divided into groups of 10 mice each. The mice in polysaccharide groups were given polysaccharides (BIWP2, BIK2, BIK10 or BIK30) orally at various doses of 10, 50 and

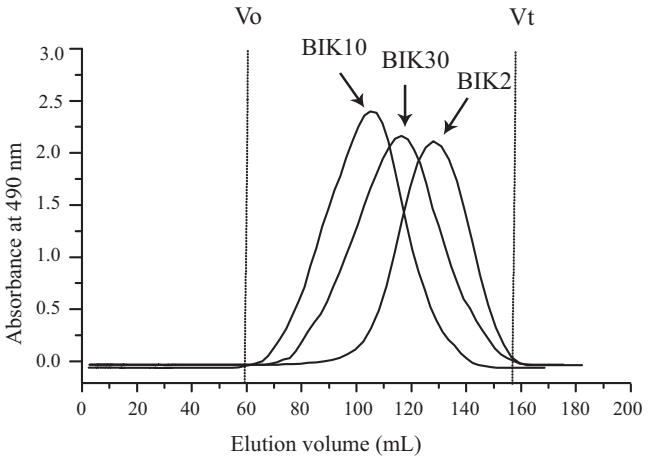

Fig. 1. Gel filtration profiles on Sepharose CL-6B (1.5 cm × 100 cm) of (1 → 6)-β-D-glucans from *B. inquinans* (Fries).

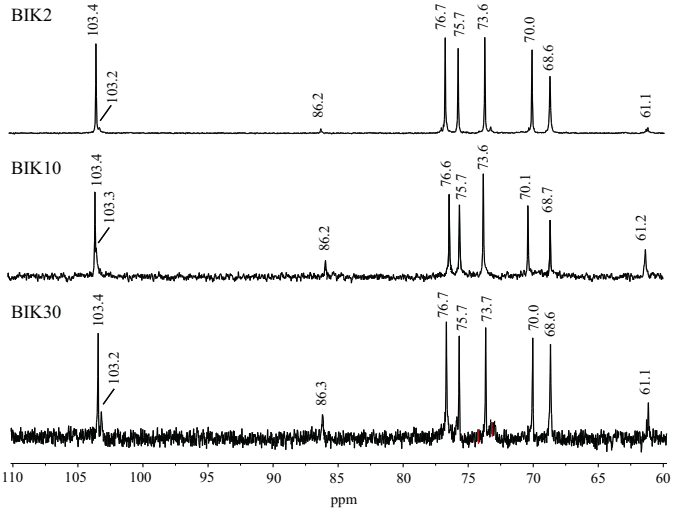

Fig. 2. ^{13}C NMR spectra of (1 → 6)-β-D-glucans from *B. inquinans* (Fries).

100 mg/kg body weight, respectively. The mice in control group were administrated equivalent volume of physiological saline. The dose volume was 0.2 mL. After treated with polysaccharides for 10 days, the mice were sacrificed. Their spleens were aseptically extirpated, and the splenocytes were prepared for the lymphocyte proliferation assay. The lymphocyte proliferation assay was performed as our previous procedure (Bi et al., 2011). Each experiment was performed in triplicate.

All the data were expressed as means ± standard deviation (S.D.) of three replications, and examined for their statistical significance of difference with Student's *t*-tests. Statistical significance was declared at $P < 0.05$.

3. Results and discussion

3.1. Isolation, purification and structural analysis of β-(1 → 6)-glucans from B. inquinans (Fries)

The fruit bodies of *B. inquinans* (Fries) were extracted by hot water and purified as our previous described procedure (Bi et al., 2009, 2011). There are two homogenous polysaccharide fractions isolated from *B. inquinans* (Fries), one is a heteropolysaccharide composed of mannose, glucose and galactose, the other is an unbranched (1 → 6)-β-D-glucan with Mw 2.6 kDa, namely BIWP2. The water-extracted residue was submitted to a successive alkaline extraction with the water solution of 2%, 10% and 30% KOH in the present study, and finally generated fractions BIK2 (1.1% yield of dried residues), BIK10 (5.1% yield of dried residues), BIK30 (3.8% yield of dried residues), respectively.

BIK2, BIK10 and BIK30 were brown powders, and respectively exhibited a single and symmetrical peak on Sepharose CL-6B gel filtration chromatography, indicating their homogeneities (Fig. 1). The monosaccharide composition analysis showed that they were all mainly composed of glucose (>92%) and a trace amount of mannose and galactose, implying they were glucans. Their sugar contents, specific rotations, molecular weights and monosaccharide compositions were listed in Table 1. As seen in Table 1, the high negative rotation suggested the dominating presence of β-glycosidic linkages in them (Dong, Yao, Yang, & Fang, 2002; Nandan et al., 2008).

Methylation analysis produced three partially methylated alditol acetates from BIK2, BIK10 and BIK30, respectively. The main product was 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl glucitol, and the others were 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl and 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl glucitol. The results of methylation analysis are summarized in Table 2. Thus the three fractions were found to contain units of (1 → 6)-, (1 → 3,6)-glucopyranosyl residues and terminal residues of D-glucopyranosyl residues at different molar ratio.

BIK2, BIK10 and BIK30 all showed abundance HIO_4 consumptions. They respectively consumed 1.93 mol, 1.69 mol and 1.51 mol periodate per mol of glucosyl residual on average, and produced 0.98 mol, 0.81 mol and 0.73 mol formic acid per mol of glucosyl residual on average. The consumptions of HIO_4 in the reactions were all about two times than the amounts of formic acid that was produced by periodate treatment, indicating the existing of 1 → or 1 → 6 linked glucosyls.

The periodate-oxidized products were hydrolyzed and analyzed by gas chromatography. The hydrolysate of BIK2, BIK10 and BIK30 all contained glucose and glycerol, and no erythritol. The molar ratios of glucose and glycerol were respectively 1:84.7 (BIK2), 1:5.4 (BIK10) and 1:3.1 (BIK30). The presence of glucose indicated that (1 → 3, 6)-linked glycosyl residues exist in these polysaccharides and this result was completely in accordance with the observation of methylation analysis. Moreover, there was no monosaccharide in the sacks, indicating that the backbones of BIK2, BIK10 and BIK30 should be all oxidized by HIO_4. So it is deduced that the backbones are composed of (1 → 6) linked glucosyls. According to the absence of erythritol and the presents of glucose and glycerol out of sacks, their backbones are substituted by single glucosyl at C-3 positions.

The NMR analyses were carried out to further characterize the structures of BIK2, BIK10 and BIK30 (Table 3). According to the NMR data in the literatures (Corradi da Silva et al., 2008; Monteiro et al., 2000; Nandan et al., 2008; Ukawa, Ito, & Hisamatsu, 2000), anomeric signals for typical β-glycosidic configurations appeared at δ 103.2–103.4 ppm in the ^{13}C NMR spectra (Fig. 2), which are consistent with the values of specific rotation. The signals of O-substituted C-6 appeared at δ 68.6 and 68.7 ppm, and those of unsubstituted C-6 appeared at δ 61.1 and 61.2 ppm. The signals at δ 86.2, 86.3 ppm were assigned to O-substituted C-3, and those of unsubstituted C-3 appeared at δ 76.6 and 76.7 ppm. The ratios of the signals from NMR are consistent with those of sugar residues characterized by the chemical analysis above.

Summarizing the comprehensive results, it is deduced that fractions BIK2, BIK10 and BIK30 are all (1 → 6)-β-D-glucans with different branching ratios. Their backbones consist of (1 → 6)-linked β-glucopyranosyl residues, and the side chains contain single β-glucosyl attached to the O-3 of glucosyls on main chains.

Table 1
The yields, total carbohydrate and protein contents, and monosaccharide compositions of (1 → 6)-β-D-glucans from *B. inquinans* (Fries).

Fraction	Yield[a] (%)	Sugar content (%)	α_D^{20} [b]	Mw (kDa)	Monosaccharide composition (%)		
					Mannose	Glucose	Galactose
BIK2	1.1	98.6	−104.7	37.5	1.9	95.7	2.4
BIK10	5.1	97.2	−136.3	288.9	3.5	92.2	4.3
BIK30	3.8	96.4	−124.1	175.5	3.0	93.4	3.6

[a] Yield calculated based on dried residues.
[b] The specific rotation was calculated at *c* 0.2 in 0.5 M NaOH.

Table 2
Methylation analysis of (1 → 6)-β-D-glucans from *B. inquinans* (Fries).

Components[a]	Molar ratio[b]			Mass fragments (*m/z*)	Linkage type
	BIK2	BIK10	BIK30		
2,3,4,6-Me_4-Glc	1.04	1.14	1.09	45, 101, 117, 129, 145, 161, 205	Glc*p*-(1 →
2,3,4-Me_3-Glc	83.7	4.11	1.93	43, 87, 99, 101, 117, 129, 161, 189, 233	→ 6)-Glc*p*-(1 →
2,4-Me_2-Glc	1.00	1.00	1.00	43, 87, 117, 129, 139, 159, 189, 233	→ 3,6)-Glc*p*-(1 →

[a] 2,3,4,6-Me_4-Glc = 1,5-tri-acetyl-2,3,4,6-tri-O-methyl glucitol, etc.
[b] Relative molar ratio, calculated from the ratio of peak areas.

Table 3
^{13}C NMR chemical shifts of (1 → 6)-β-D-glucans from *B. inquinans* (Fries).

Fraction	Glycosidic linkage	Chemical shift (δ, ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
BIK2	→ 6)-β-Glc*p*-(1 →	103.4	73.6	76.7	70.0	75.7	68.6
	→ 3,6)-β-Glc*p*-(1 →	103.4	73.6	86.2	70.0	76.7	68.6
	β-Glc*p*-(1 →	103.2	73.6	76.7	70.0	75.7	61.1
BIK10	→ 6)-β-Glc*p*-(1 →	103.4	73.6	76.6	70.1	75.7	68.7
	→ 3,6)-β-Glc*p*-(1 →	103.4	73.6	86.2	70.1	76.6	68.7
	β-Glc*p*-(1 →	103.3	73.6	76.6	70.1	75.7	61.2
BIK30	→ 6)-β-Glc*p*-(1 →	103.4	73.7	76.7	70.0	75.7	68.6
	→ 3,6)-β-Glc*p*-(1 →	103.4	73.7	86.3	70.0	76.7	68.6
	β-Glc*p*-(1 →	103.2	73.7	76.7	70.0	75.7	61.1

Base on the above results, the deduced structures of BIK2, BIK10 and BIK30 are shown in Fig. 3.

3.2. Lymphocyte proliferation activities of β-D-glucans from B. inquinans (Fries)

Lymphocytes are considered to be one of the important components of the host defense against tumor growth and invading pathogens. These cells are able to produce many kinds of cytokines after differentiation and activation. Stimulating proliferation of lymphocytes results in an increase in cytokine release, accounting for the antitumor activity of polysaccharides (Hines, Pacheco, & Maier, 2012; Leung, Liu, Koon, & Funga, 2006). As shown in Table 4, when ConA or LPS was added as mitogen for lymphocytes, all β-D-glucans could significantly increase lymphocytes proliferation ($P < 0.05$). At the dose of 50 mg/kg, unbranched glucan BIWP2 started to show a significantly lymphocytes proliferation activity, while branched glucans BIK2, BIK10 and BIK30 had significantly lymphocytes proliferation activities at the dose of 10 mg/kg in dose-dependent manners. The lymphocytes proliferation activity of BIK10 is highest in the branched glucans, but there is no significant difference between them. In order to avoid false positive results, contaminant endotoxin was tested by a gel-clot *Limulus* amoebocytes lysate (LAL) assay using an E-TOXATE kit (Sigma, St. Louis, USA) according to the manufacturer's instruction. The quantities of endotoxin in polysaccharide samples were all less than 0.015 EU/mg (negative).

BIK2: m ~ 84, n ~ 3;
BIK10: m ~ 4, n ~ 297;
BIK30: m ~ 2, n ~ 271.

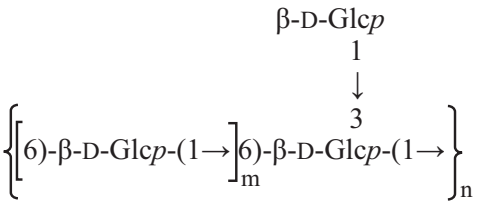

Fig. 3. Structures of (1 → 6)-β-D-glucans from *B. inquinans* (Fries). "*m*": number of (1 → 6)-linked β-glucopyranosyl residues in repeating unit; "*n*": number of repeating units in β-D-glucan.

The results of the lymphocytes proliferation activities, molecular weights and structures of the four β-D-glucans reveal that the molecular weight and the branching ratio influence immunological activities of (1 → 6)-β-D-glucans in the present experimental conditions: the (1 → 6)-β-D-glucan with higher of branching ratio and molecular weight shows higher effect on lymphocytes proliferation. Moreover, according to the effects of BIK10 and BIK30 on lymphocytes proliferation, the molecular weight of (1 → 6)-β-D-glucans should influence its immunological activity more greatly than its branching ratio. So we deduce that the molecular weight

Table 4
Immunostimulating effects of (1 → 6)-β-D-glucans on ConA- or LPS-induced lymphocyte proliferation *in vivo*.

Group	Mw (kDa)	Branching ratio	Dose (mg/kg)	Lymphocyte proliferation activity	
				ConA ($A_{570\,nm}$)	LPS ($A_{570\,nm}$)
Control				0.40 ± 0.06	0.38 ± 0.06
BIWP2			10	0.44 ± 0.02	0.41 ± 0.03
	2.6	None	50	0.48 ± 0.02[a]	0.45 ± 0.02[a]
			100	0.52 ± 0.02[a]	0.47 ± 0.01[a]
BIK2			10	0.49 ± 0.04[a]	0.43 ± 0.03
	37.5	0.01	50	0.57 ± 0.05[a]	0.50 ± 0.04[a]
			100	0.61 ± 0.01[b]	0.55 ± 0.02[b]
BIK10			10	0.51 ± 0.05[a]	0.48 ± 0.03[a]
	288.9	0.17	50	0.60 ± 0.03[b]	0.53 ± 0.02[a]
			100	0.67 ± 0.02[b]	0.62 ± 0.02[b]
BIK30			10	0.51 ± 0.02[a]	0.47 ± 0.01[a]
	175.5	0.25	50	0.59 ± 0.04[b]	0.52 ± 0.02[a]
			100	0.65 ± 0.02[b]	0.59 ± 0.03[b]

The control group was only treated with physiological saline for 10 days. Each value represents the mean ± S.D.
[a] The significant values are indicated by $P < 0.05$.
[b] The significant values are indicated by $P < 0.01$.

and the branching ratio influence the binding capacity between (1 → 6)-β-D-glucans and β-glucan receptor, and molecular weight is more effectively than branching ratio.

The major polysaccharide BRMs derived from fungi are β-D-glucan and α-D-mannan (Leung et al., 2006). β-D-Glucan is mainly obtained from the cell wall of chitinous fungi, which has chitin as the major fibrils of cell wall (Bartnicki-Garcia, 1968). To the best of our knowledge, the characterized pattern recognition receptors (PRRs) which recognize polysaccharide BRMs are Class A SR, β-glucan receptor, mannose receptor and complement receptor type 3 (CR3) (Brown et al., 2002; Kim, Ordija, & Freeman, 2003; Ross & Vetvicka, 1993; Zamze et al., 2002). β-Glucan receptor is widely expressed on monocytes, macrophages, DC, neutrophils, eosinophils, B- and T-lymphocytes (Brown & Gordon, 2001; Brown, 2006; Willment et al., 2005). Like some other bioactive (1 → 6)-β-D-glucans isolated from fungi (Haladova et al., 2011; Han, Chai, Jia, Han, & Tu, 2010; Mizuno et al., 1990; Zhang, Cui, Cheung, & Wang, 2007), (1 → 6)-β-D-glucans from *B. inquinans* (Fries) could significantly increase the ConA or LPS-induced lymphocytes proliferation *in vivo*, and might be potent T/B-cell stimulators *via* β-glucan receptor.

4. Conclusion

Previously, an unbranched (1 → 6)-β-D-glucan (BIWP2) was extracted from the fruit bodies of *B. inquinans* (Fries) by hot water. In this paper, three polysaccharides were isolated from the water-extracted residues by a successive alkaline extraction with the water solution of 2%, 10% and 30% KOH, and characterized to be branched (1 → 6)-β-D-glucans. Their backbones are substituted by single glucosyls at C-3 positions, the branching ratios are respectively 0.01 (BIK2), 0.17 (BIK10), 0.25 (BIK30). The immunological tests showed that all the four β-D-glucans from *B. inquinans* (Fries) could significantly increase the ConA or LPS-induced lymphocytes proliferation *in vivo*. Moreover, branched glucans (BIK2, BIK10 and BIK30) had more significantly lymphocytes proliferation activities than unbranched glucan (BIWP2), and the effect of (1 → 6)-β-D-glucans on lymphocytes proliferation increased along with the molecular weight. Therefore, the (1 → 6)-β-D-glucans from *B. inquinans* (Fries) are possible potential immunostimulating agents for use in healthcare food or medicine, and the structure–activity relationships of (1 → 6)-β-D-glucans are well enriched by the present results.

Acknowledgements

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