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An antioxidative galactomannan–protein complex isolated from fermentation broth of a medicinal fungus Cs-HK1

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ABSTRACT

A protein-containing polysaccharide, EPS2BW, was fractionated from the exopolysaccharide (EPS) produced by a medicinal fungus *Cordyceps sinensis* (Cs-HK1). EPS2BW was mainly composed of galactomannan with about 16% (w/w) protein and 50 kDa average molecular weight. The galactomannan part consisted of mannose and galactose at 1.7:1.0 molar ratio, and the protein segments were composed of sixteen amino acids with 12.5% proline and 16.6% threonine (mol%) being the most abundant. Based on analytical results from NMR, methylation analysis, partial acid hydrolysis and GC–MS, the galactomannan structure was elucidated as a (1 → 2)-α-D-mannopyranosyl (Man*p*) backbone with *O*-6-linked galactopyranosyl (Gal*p*) branches. EPS2BW exhibited a high antioxidant capacity in both chemical and cell culture assays, with a Trolox equivalent radical scavenging activity of 44.7 μmol Trolox/mg, a Fe^{3+} reducing power of 38.9 μmol Fe^{2+}/mg, and significant cytoprotective effect against H_2O_2-induced PC12 cell death at 50–250 μg/mL.

1. Introduction

Cordyceps sinensis (Berk.) Sacc., the Chinese caterpillar fungus or Dong-Chong-Xia-Cao in Chinese, is a rare and precious medicinal fungus which has been used in traditional Chinese medicine for a range of health promoting functions (Li et al., 2003; Shashidhar, Giridhar, Udaya Sankar, & Manohar, 2013). Previous studies have demonstrated several pharmacological and bioactive effects of the fungus such as anticancer, immunomodulation, antioxidant, hypolipidemic and hypoglycemic (Zhou, Gong, Su, Lin, & Tang, 2009). Excessive production of free radicals and reactive oxygen species (ROS) in human body causes oxidative stresses and adverse effects to human health (Ray, Huang, & Tsuji, 2012). As radicals and ROS are constantly produced by enzymatic reactions in cells, antioxidant protection against these highly reactive species is vital for maintaining the integrity of cellular structures and biomolecules (De la Fuente, 2002). *C. sinensis* fungus has been well-known for its protective effects on human organs, such as liver, kidney and heart (Zhou et al., 2009) and some of the fungal extracts have also shown significant antioxidant activities (Yamaguchi, Kagota, Nakamura, Shinozuka, & Kunitomo, 2000; Tan et al., 2011).

Polysaccharides (PS) and their complexes with proteins (PSPs) represent a class of the most abundant bioactive constituents of *C. sinensis* and other medicinal fungi. More than 35 years ago, a galactomannan was isolated from the fruit bodies of *C. sinensis* and its structure was elucidated as of a (1 → 2)-α-D-Man*p* main chain (Miyazaki, Oikawa, & Yamada, 1977). Another galactomannan containing a minor protein content was later isolated from the fruit bodies of *C. sinensis*, having a (1 → 2)-α-D and (1 → 6)-α-D-Man*p* main chain (Kiho, Tabata, & Ukai, 1986). Galactomannans have been used as food stabilizers (Cerqueira et al., 2011) and diagnostic agents for invasive aspergillosis in immunocompromised patients (Pfeiffer, Fine, & Safdar, 2006). Very recently, a galactomannan from guar gum has been shown safe to be used as a clinical grade galectin antagonist for cancer patients (Demotte et al., 2014).

As natural *C. sinensis* is very rare and not sufficient to meet the increasing demand in recent years, mycelial fermentation has become a dependable measure for production of the fungal materials as the major source of PS and other useful components. A number of PS structures have been reported, which were mostly isolated and purified from the water or alkaline extracts of mycelial biomass produced by solid or liquid fermentation (Zhou et al., 2009; Yan, Wang, & Wu, 2014). In addition to extraction of (intracellular) PS from the mycelia biomass, exopolysaccharides (EPS) can be produced by some fungal species and isolated from the liquid fermentation broth. Cs-HK1 is a fungal species isolated from natural

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C. sinensis fruiting body in our lab and Cs-HK1 mycelial culture has been established, and applied to liquid fermentation for production of fungal mycelia and EPS (Yan et al., 2014). The crude EPS from the Cs-HK1 mycelial culture was a complex mixture of PS and PSP complexes in a wide molecular weight (MW) range from ~5 kDa to over 1000 kDa, and the PSPs with a higher protein content have shown more significant antioxidant activities (Leung, Zhao, Ho & Wu, 2009; Chen, Siu, Wang, Liu, & Wu, 2013).

Up to date, no galactomannan has been retained from the fungal mycelia or EPS produced by *C. sinensis* fermentation. This study was to analyze the chemical composition and elucidate the molecular structure of a novel galactomannan–protein complex fractionated, and purified from the EPS produced by the Cs-HK1 fungus in liquid fermentation. Its antioxidant capacity was evaluated by chemical and cell culture assays.

2. Materials and methods

2.1. Cs-HK1 mycelial fermentation for EPS production

The Cs-HK1 fungus used in this study was originally isolated from a natural *C. sinensis* fruiting body-caterpillar complex and routinely maintained in our lab on solid PDA medium at 20 °C as reported previously (Leung, Zhang, & Wu, 2006). Liquid culture was initiated by inoculating the Cs-HK1 fungus from the solid culture into a flask of liquid medium, which was composed of 40 g glucose, 10 g yeast extract, 5 g peptone, 1 g KH_2PO_4 and 0.5 g $MgSO_4{\cdot}7H_2O$ (per liter), followed by incubation on a rotary shaker at 150 rpm and 20 °C for 6–7 days. The mycelial culture broth was then inoculated into several 1-L Erlenmeyer flasks each containing 200 mL of the same liquid medium to start the liquid fermentation for EPS production. After 7-days of shaking incubation, the mycelial fermentation liquid was centrifuged at 14,000 rpm for 15 min and the supernatant liquid broth was collected for EPS isolation.

2.2. Isolation and fractionation of EPS from fermentation broth

The EPS was isolated from the solid-free fermentation broth by ethanol precipitation in two steps (using 95% grade ethanol) as reported previously (Chen, Siu, Cheung, & Wu, 2014). The first step was performed with an ethanol volume ratio of 2/3 (~40% v/v) to precipitate the higher MW EPS fraction (EPS1). In the second step (after the removal of EPS1), the remaining supernatant using an ethanol volume ratio of 1:1 (~50% v/v) to precipitate the lower MW EPS fraction, named EPS2 with a yield of 0.2 g/L. In each step, ethanol was added slowly to the EPS solution with stirring, and then left undisturbed on the bench at room temperature for overnight (~12 h). The precipitate was recovered by centrifugation (10,000 rpm, 20 min) and lyophilized, and collected as the crude EPS fractions for further fractionation and analysis.

The lower MW EPS2 (~400 mg) from above was dissolved in 2 mL distilled water and loaded to a DEAE-Sephadex A-25 column (2.6 × 100 cm) (Pharmacia), and eluted stepwise with distilled water and 0.1 M NaCl at a flow rate 0.5 mL/min. Polysaccharide in the elute was detected by the anthrone agent (Sigma). A major EPS fraction was collected during distilled water elution and treated with 15% trichloroacetic acid (TCA) at 4 °C to remove protein, yielding the EPS fraction EPS2BW (~80 mg).

2.3. Analysis of molecular weight

The MW distribution and homogeneity of EPS2BW were analyzed by high performance gel permeation chromatography (HPGPC) with two columns in series, Ultrahydrogel™ 2000 and Ultrahydrogel™ 500 column (Waters). The HPGPC was performed with a Waters HPGPC instrument with a Waters 1515 isocratic HPLC pump, a Waters 2414 refractive index (RI) detector and a Waters 2998 UV detector. The columns were equilibrated and eluted with mobile phase containing 2.9 g/L $NaH_2PO_4.2H_2O$ and 11.5 g/L Na_2HPO_4 at a flow rate of 0.6 mL/min. The HPGPC was calibrated (MW versus elution time) with several dextran standards ranging from 1 to 670 kDa (Sigma-Aldrich, USA).

2.4. Analysis of chemical composition

The total carbohydrate content was determined by the anthrone test using glucose as a standard and the protein content by the Lowry method using bovine serum albumin (BSA) as a standard as reported previously (Leung et al., 2009; Chen et al., 2013).

Monosaccharide composition was analyzed by 1-phenyl-3-methyl-5-pyrazolone (PMP)-HPLC method (Honda et al., 1989) with minor modifications, as described previously (Chen et al., 2013). In brief, the EPS sample (1–2 mg) was completely hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h. The hydrolysate was dried under vacuum, and then derivatized with 450 μL PMP solution (0.5 M in methanol) and 450 μL of 0.3 M NaOH at 70 °C for 30 min. The reaction was stopped by neutralization with 450 μL of 0.3 M HCl, followed by extraction with chloroform (1 mL, 3 times). The extract solution was applied to HPLC analysis with an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm) on an Agilent 1100 instrument with a G1312A Binpump and a UV detector.

Amino acid analysis was performed as described by Simpson, Neuberger and Liu (1976). Briefly, hydrolysis of sample was carried out in heavy walled ignition tubes which had been washed with a mixture of H_2SO_4/HNO_3 (3:1), rinsed in deionized H_2O, and oven-dried. EPS2BW (2 mg) was hydrolyzed in vacuo at 115 °C for 72 h with 1.0 mL of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole. The hydrolysates were partially neutralized with 1.0 mL of 3.5 M NaOH, and filtered for amino acid analysis. The analysis was performed on an Agilent Series 1100 instrument with a ZORBAX Eclipse-AAA column (3.5 μm, 3.0 × 150 mm) and a fluorescence detector.

2.5. Partially acid hydrolysis and methylation analysis

EPS2BW (50 mg) was partially hydrolyzed with 0.1 M TFA at 100 °C for 1 h. The acid was removed under reduced pressure by repeated evaporation with methanol followed by dialysis (3,500 Da MWCO). Both the retentate (designated EPS2BW-I: 27 mg) and the dialysate (EPS2BW-O: 22 mg) were collected, concentrated and lyophilized, and subjected to composition and methylation analysis. EPS2BW and its partially hydrolyzed fractions were methylated three times as described by Needs and Selvendran (1993) with minor modifications. In brief, 10 mg of EPS sample was dissolved in 3.0 mL dimethyl sulfoxide (DMSO), followed by the addition of 200 mg powdered NaOH and sonication (for mixing) in an ultrasonic bath for 10 min. After incubation for 10 min at room temperature with stirring, 1 mL of iodomethane was added slowly to the reaction solution. The reaction mixture was placed in darkness for 1 h, followed by the addition of 2.0 mL distilled water. The methylated EPS was extracted with 2 mL chloroform three times and dried in a rotary evaporator at reduced pressure, and collected as the methylated alditol acetates for linkage analysis by GC–MS which was performed with an Agilent 6890 N-5973 instrument.

2.6. NMR and IR spectroscopy

A Bruker AV400 instrument was used for the NMR. For NMR spectrum analysis, the EPS sample (30 mg) was co-evaporated with D_2O twice by lyophilization, and then dissolved in 600 μL D_2O containing 0.1 μL acetone as an internal reference. Infrared (IR) spectroscopy of EPS was performed at room temperature in the

range of 4000–500 cm^{-1} at 4 cm^{-1} resolution on an Avatar 360 FTIR (Thermo Nicolet, Cambridge, UK). All spectra were baseline corrected.

2.7. Antioxidant activity assays

The antioxidant capacity of EPS2BW was evaluated with two chemical assays, the Trolox equivalent antioxidant capacity (TEAC) and the ferric reducing ability of plasma (FRAP), and a cell culture assay on cytoprotective activity against oxidative stress. The TEAC and FRAP assays were performed with the procedures and conditions as reported in details by Leung et al. (2009). In brief, the EPS sample was predissolved in distilled water and added to the assay reagent solution at a suitable final concentration, and incubated at a set temperature over 24 h, during which sample was taken at 2 h, 12 h and 24 h for measurement of absorbance at 734 nm for TEAC or 593 nm for FRAP assay. The radical scavenging activity value at each time point was calculated by $(1 - A/A_0) \times 100\%$, where A and A_0 are the absorbance values of $ABTS^{\bullet+}$ or Fe^{3+} solution in the presence and absence of the polysaccharides sample, respectively, or the antioxidant reference (Trolox for TEAC assay or Fe^{2+} for FRAP assay). The activities were converted to TEAC (μmol Trolox/mg sample) by the calibration with Trolox from 0 to 60 μmol or FRAP (μmol Fe^{2+}/mg sample) by the calibration with Fe^{2+} from 0 to 50 μmol, respectively.

The cytoprotective activity test was performed in rat pheochromocytoma PC12 cell culture, subjected to peroxide H_2O_2 treatment as described by Chen et al. (2013). The PC12 cell culture was routinely propagated in RPMI1640 medium, which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, and incubated in humidified, 5% CO_2 atmosphere at 37 °C. Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For the activity test, the EPS2BW sample was pre-dissolved in phosphate buffered saline (PBS) at 10 mg/mL and the H_2O_2 solution was freshly prepared at 100 mM in PBS. In brief, 5000 of PC12 cells were seeded into 96-well microplate in phenol red free RPMI 1640 medium. After 24 h, H_2O_2 (80 μM) was added to the wells and incubated for 4 h, and followed by the addition of the EPS solution at 3.9–500 μg/mL final concentrations and incubation for another 24 h. The cell viability was determined by MTT (Mosmann, 1983). A cytotoxicity test was also performed on EPS2BW in the PC12 cell culture, with the same procedure and conditions as for the cytoprotective activity test, but without the exposure to H_2O_2.

3. Results and discussion

3.1. MW distribution and chemical composition of EPS2BW

EPS2 was eluted with distilled water by DEAE-Sephadex A-25 column (Supplemental Fig. 1a). The major peak was collected, and deprotainized (by TCA) to give EPS2BW. EPS2BW exhibited a symmetrical peak on the HPGPC detected by RI and UV detector (Fig. 1a), indicating its molecular homogeneity. The average MW of EPS2BW was estimated as 50 kDa. EPS2BW had total carbohydrate content of 74.3% and a protein content of 15.8% (carbohydrate to protein ratio 4.7:1.0). HPLC analysis of the completely hydrolysed products with 2 M TFA showed that EPS2BW consisted of mannose and galactose at the molar ratio of 1.7:1.0 (Supplemental Table 1). As for the two partially hydrolyzed products with 0.1 M TFA, EPS2BW-I (the retentate of dialysis) consisted of mannose and galactose at 7.2:1.0 molar ratio detected by HPLC; the other, EPS2BW-O (the dialysate), was composed of ~34% total carbohydrate and ~46% protein and its carbohydrate part consisted of mannose and galactose in the ratio

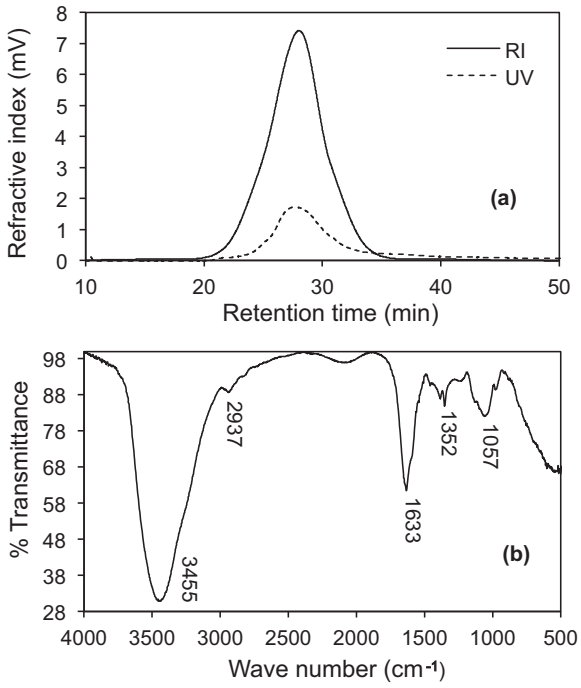


Fig. 1. HPGPC spectrum (a) and IR spectrum (b) of EPS2BW.

of 1.0:8.9 (Supplemental Table 1). From GPC and composition analysis, EPS2BW was deduced as a PS-protein complex or probably a proteoglycan, though the analytical results could not give a clue for the bonding position of protein to the PS chain.

The amino acid composition of the protein part in EPS2BW was shown in Table 1. Table 1 also showed the amino acid composition of a protein–galactomannan (CT-4N) isolated from the fruit body of *C. sinensis* by Kiho et al. (1986). Interestingly, the two had a very similar amino acid composition in the amino acid constituents and relative content.

3.2. Glycosidic linkages of EPS2BW from methylation analysis

Table 2 shows the results from methylation analysis of EPS2BW and its two hydrolysates. The intra-chain residues contained (1 → 2)-linked Man*p* (28.2%) and (1 → 3)-linked Gal*p* (3.4%). The non-reducing terminals consisted of Gal*p* (30.3%) and a small amount of Man*p* (3.6%), indicating that EPS2BW was highly

Table 1
Amino acid composition of the protein segments in EPS2BW (mol%).

Amino acid	EPS2BW	CT-4N[a]
Aspartic acid	7.9	6.3
Glutamic acid	6.8	13.3
Serine	8.0	11.8
Histidine	1.8	0.8
Glycine	5.5	12.9
Threonine	16.6	10.7
Arginine	4.7	1.2
Alanine	8.3	14.2
Tyrosine	1.3	0.5
Cysteine	0.6	1.5
Valine	8.3	8.4
Phenylalanine	2.5	1.4
Isoleucine	4.0	2.5
Leucine	3.5	2.6
Lysine	7.7	1.4
Proline	12.5	10.5

[a] Kiho et al. (1986).

Table 2
Linkage analysis of EPS2BW and its partial acid hydrolysis products.

Methylated sugars	Linkages	Molar ratios (%)		
		EPS2B	EPS2B-I	EPS2B-O
2,3,4,6-Me$_4$-Man*p*	T-Man*p*	3.6	20.4	7.5
2,3,4,6-Me$_4$-Gal*p*	T-Gal*p*	30.3	7.1	78.4
3,4,6-Me$_3$-Man*p*	1,2-Man*p*	28.2	52.8	1.1
2,4,6-Me$_3$-Gal*p*	1,3-Gal*p*	3.4	5.9	4.4
4,6-Me$_2$-Gal*p*	1,2,3-Gal*p*	3.2	–	–
3,4-Me$_2$-Man*p*	1,2,6-Man*p*	31.3	13.8	1.0
Hexitol hexaacetate	–	–‘	–	7.6

branched, with the main branching points situated at (1 → 2,6)-linked Man*p* (31.3%) and (1 → 2,3)-linked Gal*p* (3.2%).

With one of the partially hydrolyzed fractions, EPS2BW-I, the proportion of (1 → 2)-linked Man*p* significantly increased with the decrease in T-Gal*p*, suggesting that EPS2BW had a backbone consisting mainly of a (1 → 2)-linked Man*p* residue. The ratios between (1 → 2)- and (1 → 2,6)-linked Man*p* in EPS2BW and EPS2BW-I were 0.9 and 3.8, respectively, indicating that the increase in (1 → 2)-linked Man*p* was at the expense of the (1 → 2,6)-linked Man*p* and EPS2BW contained (1 → 2,6)-linked Man*p* in the backbone. Therefore, the backbone of EPS2BW consisted both of (1 → 2,6)-linked Man*p* (31.3%) and (1 → 2)-linked Man*p* (28.2%) residues in a ratio of 1.1:1.0. The other partially hydrolyzed fraction, EPS2BW-O (Table 3), contained 78.4% T-Gal and 7.6% hexitol hexaacetate (by mole), suggesting the presence of galactose and galactobiose residues in the oligosaccharides.

3.3. IR and NMR spectral characteristics of EPS2BW

As shown in Fig. 1b, the IR peak at 3455 cm^{-1} was characteristic of O—H stretching vibration, the peak at 2937 cm^{-1} was characteristic of C—H stretching, and the peak at 1633 cm^{-1} was ascribed to C=O in protein. In addition, no peak appeared at 1250 cm^{-1} and 850 cm^{-1}, indicative of no sulfation in EPS2BW.

The ^{13}C-NMR spectrum of EPS2BW (Fig. 2a) showed anomeric carbon signals at 107.8, 107.0, 106.0, 102.2, 98.2 and 97.1 ppm, labeled A–F. The signals at 174.8 and 21.8 ppm were attributed to the C=O and alkyl groups of protein. Compared with the ^{13}C-NMR spectrum of EPS2BW, some signals disappeared in the ^{13}C-NMR spectrum of EPS2BW-I (Fig. 2b). The disappearance of protein signals suggested that protein was lost after the partial acid hydrolysis. This result was consistent with that from total protein content analysis, indicating that EPS2BW-I consisted purely of carbohydrates. The signals at 107.8, 107.0 and 105.9 ppm also disappeared after partial acid hydrolysis. Together with the linkage analysis, these three signals are attributed to the anomeric carbon of galactose residues which were removed by partial acid hydrolysis. It was

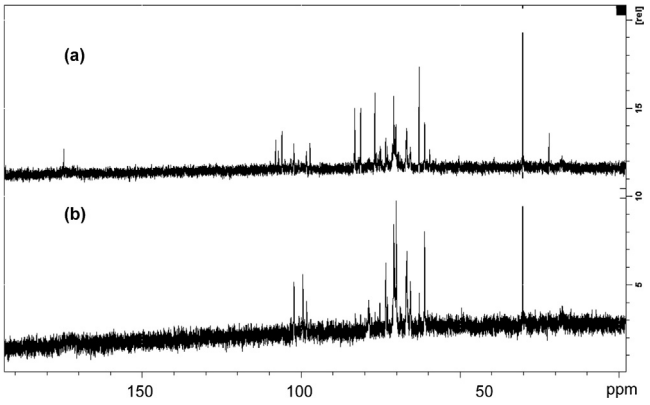


Fig. 2. ^{13}C-NMR spectrum of EPS2BW (a) and its hydrolysis product EPS2BW-I (b).

deduced that EPS2BW was a protein-containing polysaccharide with a backbone consisting of Man*p* residues and side chains of Gal*p* residues.

Fig. 3 shows the heteronuclear single- and multiple-quantum coherence spectrum (HSQC: Fig. 3a; HMQC: Fig. 3b) of EPS2BW, from which the relevant hydrogen and carbon signals were assigned (Table 3). The shift of anomeric carbon in the non-reducing terminal of β-D-Gal*p* residues is slightly higher than that in (1 → 3)-β-D-linked Gal*p* residues (Bilan, Vinogradova, Shashkov, & Usov, 2007) but lower than that in (1 → 2)-β-D-linked Gal*p* residues (Zhao et al., 2007). Therefore, the carbon signals at 107.8, 107.0 and 105.9 ppm were attributed to the anomeric carbon signals A–C (Table 3), respectively. The ratio of (1 → 2)-α-D-Man*p* residues increased while the ratio of (1 → 2,6)-α-D-Man*p* residues decreased after partial acid hydrolysis. Based on these changes in the signal strength of Man*p* residues before and after acid hydrolysis, the major anomeric carbon signals at 102.2, 98.2, 97.1 ppm were assigned to the anomeric carbon signals D–F (Table 3), respectively. Other than the major signals A–F assigned from the HSQC and HMBC spectrum, it is not possible to assign all signals of the saccharide units because of signal overlapping. The reversed resonance at 66.66 ppm in distortionless enhancement by polarization transfer (DEPT) spectrum (Supplemental Fig. 2) undoubtedly arose from C6 of (1 → 2,6)-α-D-Man*p* residues. In Fig. 3b, the cross peak at 4.98 and 66.7 ppm suggested that H1 of (1 → 2,3)-β-D-Gal*p* residues coupled with C6 of (1 → 2,6)-α-D-Man*p* residues, and (1 → 2,3)-β-D-Gal*p* residues was attached at O-6 of (1 → 2,6)-α-D-Man*p* residues. The cross peak at 5.17 and 107.84 ppm suggests that C1 of (1 → 2,3)-β-D-Gal*p* residues coupled with H1 of β-D-Gal(1→ and Gal*p* terminals was attached to (1 → 2,3)-β-D-Gal*p* residues.

Table 3
^{1}H-NMR and ^{13}C-NMR shifts of EPS2BW (δ, ppm).

Section	sugar residues	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
E	→2) -α-D-Man(1→	5.04	3.99	3.83	n.m	n.m	3.63
		98.21	70.02	70.46	n.m.	n.m.	62.73
C	→3)-β-D-Gal(1→	5.09	3.72	4.03	n.m	4.03	3.83
		105.89	74.32	82.73	n.m.	76.91	61.17
F	α-D-Man(1→	4.96	3.71	3.73	n.m	n.m	3.60
		97.08	73.42	70.89	n.m.	n.m.	62.73
A	→3)-β-D-Gal(1,2→	4.96	3.72	4.07	3.87	3.61	3.62
		107.84	74.23	81.98	70.81	72.25	59.53
B	β-D-Gal(1→	5.14	n.m	3.90	n.m.	3.97	3.56
		106.98	n.m.	82.73	n.m.	75.23	59.53
D	→6) -α-D-Man(1,2→	4.93	3.80	n.m.	n.m.	3.70	3.93
		102.15	70.80	n.m.	n.m.	72.91	66.66

n.m.: Not marked.

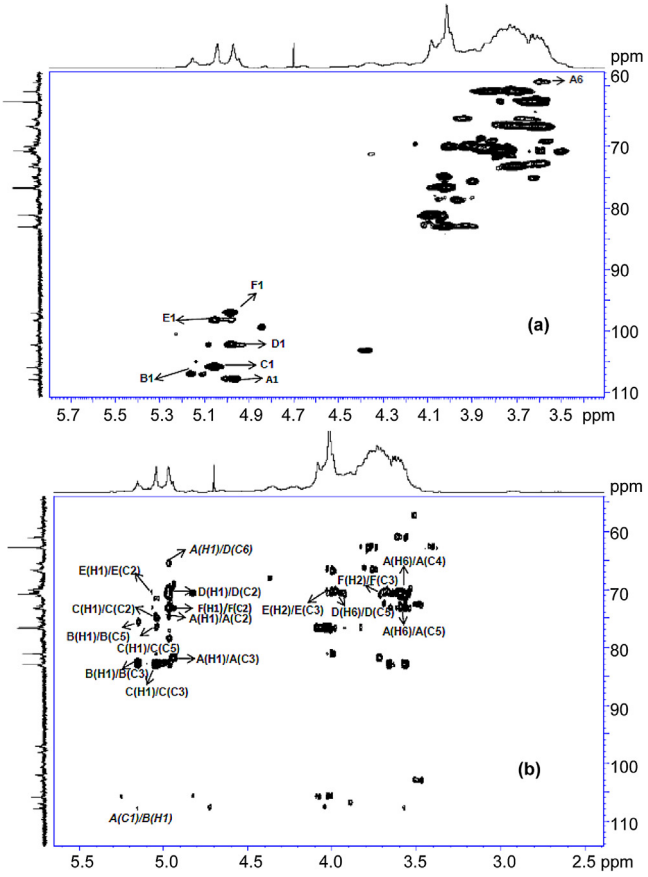
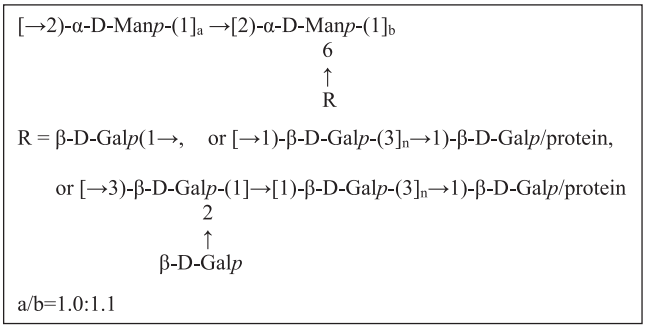


Fig. 3. HSQC spectrum (a) and HMBC spectrum of EPS2BW (b).

From all above analytical results, the structure of EPS2BW was deduced as



3.4. Antioxidant capacity of EPS2BW

Fig. 4a and b shows the TEAC ABTS$^{\bullet+}$ radical scavenging activity and the FRAP activity, respectively. In both figures, the antioxidant activity levels increased with the reaction time, significantly from 2 h to 12 h and slightly from 12 to 24 h. The TEAC and FRAP scavenging capability of EPS2BW were also dose-independent (data not shown). In a recent report (Chen et al., 2013), a few protein-containing EPS fractions with average MW from 6–30 kDa isolated from the Cs-HK1 mycelial fermentation broth have shown TEAC and FRAP activity in the ranges of 0.155–1.12 μmol Trolox/mg and 0.0095–0.611 μmol Fe^{2+}/mg, respectively. In comparison, EPS2BW has shown much higher TEAC and FRAP activity (23.0–45.0 μmol Trolox/mg and 15.0–38.9 μmol Fe^{2+}/mg, respectively), and has a stronger antioxidant capacity according to these two assays. Free radical scavenging by carbohydrates occurs by the hydroxyl radical

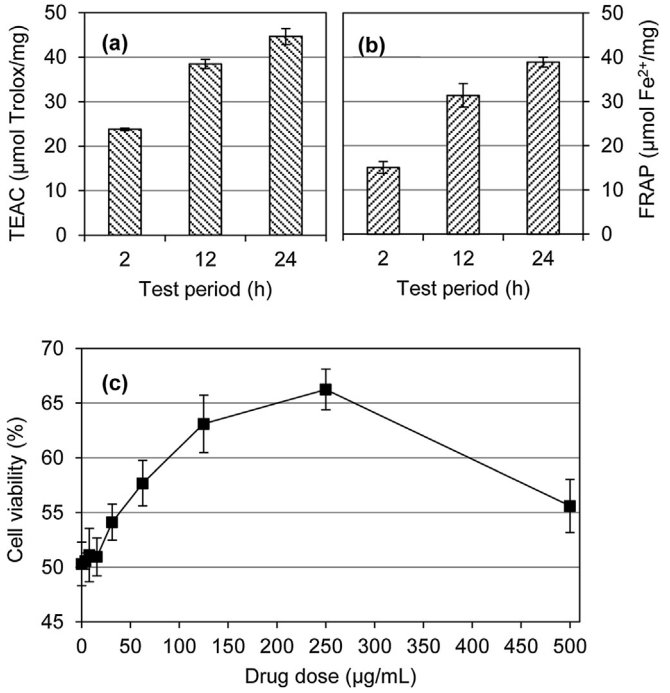


Fig. 4. Antioxidant activities of EPS2BW: (a) TEAC scavenging activity on ABTS$^{\bullet+}$ radicals; (b) FRAP activity for reducing Fe^{3+} ions; (c) Cytoprotective effects against H_2O_2-induced PC12 cell injury at various EPS2BW concentrations. The TEAC or FRAP activity value was the average (slope) at various concentrations from 100 to 1000 mg/L. For the cell culture tests, cells were treated with 80 μM H_2O_2 and EPS2BW or with an equal volume of PBS for the negative control group for 24 h. Each value was expressed as mean ± SD (n = 3).

(ABTS$^{\bullet+}$ in TEAC assay) reacting with a hydroxyl group bonded to a carbon (Elizabeth & Ana, 2012). The FRAP assay is based on reduction of ferric ions to ferrous ions by the single electronic of oxygen in hydroxyl group (Huang, Ou, & Prior, 2005). Both the two assays probably involve reactions with the hydroxyl groups in PS. Because of the large size and complex conformation of PS, the antioxidative reaction by electron-transfer reaction may be slower and lasting longer (Yanaki & Norisuye, 1983; Morris, Rees, Thoms, & Welsh, 1977). Therefore, EPS2BW may be a potential long-action antioxidant to be used in combination with the fast-action small molecule antioxidants.

The cytotoxicity test confirmed that EPS2BW had no significant inhibition or stimulation of the PC12 proliferation under normal conditions (Supplemental Fig. 3). Fig. 4c shows the cytoprotective activity of EPS2BW against H_2O_2-induced PC12 cell death (decrease of viability) at different concentrations. Pretreatment of the PC12 cells with EPS2BW at suitable concentrations (31–250 μg/mL) protected the PC12 from H_2O_2 (80 μM) damage in a dose dependent manner, retaining a higher cell viability from 54% to 66.2% than the control of 50% viability. The protective effect was dropped when the treatment dose was increased from 250 to 500 μg/mL (55.6% viability) due probably to the adverse effect on the cells at a high concentration.

A large number of PS with various structures and characteristics has been documented of the *C. sinensis* fungi, including a few α- or β-glucans and PSPs, and various heteropolysaccharides (Yan et al., 2014). Some of these PS and PSPs have exhibited various activities, hypoglycemic, antioxidant antitumor, and immuno-activities. Most of the heteropolysaccharides are composed of glucose, mannose and galactose, such as mannoglucans, galactomannans, and glucomannogalactans. A majority of these PS and PSPs are isolated from the mycelium biomass, some from the liquid fermentation broth (as EPS) of various *C. sinensis* fungi, and only a few from

the fruit bodies, e.g. a galactomannan (Miyazaki et al., 1977) and a protein-containing galactomannan CT-4N (Kiho et al., 1986). In present study, the EPS2BW purified from the EPS of Cs-HK1 fermentation was a galactomannan–protein complex with similar composition and chain linkage to that of the CT-4N from the fruit body of *C. sinensis* (Kiho et al., 1986).

4. Conclusions

EPS2BW, a galactomannan–protein complex about 50 kD, has been fractionated from the lower-MW EPS produced by liquid fermentation of Cs-HK1 fungal mycelia. EPS2BW has shown a strong antioxidant capacity in both chemical and cell culture assays. The composition of carbohydrates and amino acids in EPS2BW as well as its galactomannan backbone showed a high similarity to a protein–galactomannan isolated many years ago from the fruit bodies of *C. sinensis*. Our present study was perhaps the first to recover and elucidate the structure of a galactomannan–protein complex as a fraction of the EPS produced by a *C. sinensis* fungus in liquid fermentation. This study has again demonstrated that mycelial fermentation is a feasible process for production of bioactive polysaccharides and their protein complexes with similar structures and biological functions to those from the natural *C. sinensis* fruiting bodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.06.021.

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