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Elucidation of the structure of a bioactive hydrophilic polysaccharide from *Cordyceps sinensis* by methylation analysis and NMR spectroscopy

Shao-Ping Nie[a,b], Steve W. Cui[a,b,*], Aled O. Phillips[c], Ming-Yong Xie[a], Glyn O. Phillips[d,e], Saphwan Al-Assaf[d,e], Xiao-Liang Zhang[f]

[a] *State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China*
[b] *Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ont., Canada N1G 5C9*
[c] *Institute of Nephrology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN Wales, UK*
[d] *Glyn O. Phillips Hydrocolloids Research Centre, Glyndwr University, Wrexham, LL11 2AW Wales UK*
[e] *Phillips Hydrocolloids Research Ltd, 45 Old Bond Street, London W1S 4AQ, UK*
[f] *Institute of Nephrology, Zhong Da Hospital, Southeast University, Nanjing, PR China*

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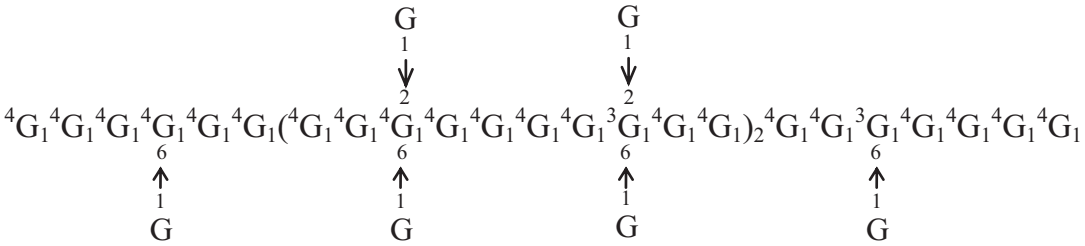
ABSTRACT

The structure of a bioactive hydrophilic polysaccharide fraction from *Cordyceps sinensis* (CBHP) was studied using methylation analysis and 2D NMR spectroscopy. Monosaccharide composition analysis revealed that it consists mainly of glucose (95.19%) with trace amounts of mannose (0.91%) and galactose (0.61%). The results of methylation analysis indicated that α-1,4 linked Glc*p* is the main linkage type (65.7%), followed by t-Glc*p* (20.7%), 1,2,3,6-Glc*p* (4.1%), 1,2,4,6-Glc*p* (3.0%), 1,3,6-Glc*p* (2.0%), 1,4,6-Glc*p* (1.6%), and 1,2-Man*p* (1.9%) and 1,3-Gal*p* (1.0%). Based on 1D and 2D NMR analysis, a preliminary structure is proposed: The backbone is composed of Glc*p* joined by 1 → 4 linkages and 1 → 3 linkages; the branching points are located at O-2 or O-6 of Glc*p* with α- terminal-D-Glc*p* as side chain. The trace amounts of 1,2-Man*p* and 1, 3-Gal*p* linkages are probably located randomly in the side chains. A schematic structure is proposed as following:



1. Introduction

Cordyceps sinensis, a well-known and valued traditional Chinese medicine, is also called "DongChongXiaCao" (winter worm summer grass) in Chinese and is also known as "Tochukaso" in Japanese, consists of the dried fungus *C. sinensis* growing on the larva of the caterpillar. The parasitic complex of the fungus is found in the soil of the prairie at an elevation of above 2000 m on the Qinghai-Tibetan plateau. Chinese early medical text listed the traditional usage of *Cordyceps* as going to the Lung and Kidney meridian and being useful as a "Lung Protectorate", for "Kidney Improvement" and as a "Yin/Yang double invigorant" (Zhu, Halpern, & Jones, 1998a, 1998b). Modern researches have revealed that *C. sinensis* possesses numerous beneficial effects for human health, including enhancing and regulating immune functions, anti-aging, anti-tumour, anti-microbial, antioxidant activity, treatment and protection against renal toxicity, heart diseases, and liver disease, respiratory disease, etc. (Bok, Lermer, Chilton, Klingeman, & Towers, 1999; Ji et al., 2009; Li et al., 2006; Rao, Fang, & Tzeng, 2007; Shi et al., 2009; Yu, Wang, Huang, & Duh, 2006). These beneficial effects have been

* Corresponding author at: Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ont., Canada N1G 5C9.
Tel.: +1 519 780 8028; fax: +1 519 829 2600.
E-mail addresses: cuis@agr.gc.ca, Steve.Cui@AGR.GC.CA (S.W. Cui).

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partly attributed to its variety of chemical ingredients, including nucleosides (cordycepin), polysaccharides, alkaloids, amino acids, inorganic elements, etc. However, nucleosides, alkaloids, mannitol, amino acids and various enzymes have always been the main focus of these studies (Huang, Liang, Guo, Zhou, & Cheng, 2003; Li et al., 2004, 2006; Yoshikawa et al., 2007; Yuan, Wang, Liu, Kuang, & Zhao, 2007; Zhu et al., 1998a, 1998b).

In recent years, many herbs and mushrooms have been reported to contain polysaccharides with a variety of biological activities (Zhang, Cui, Cheung, & Wang, 2007). It has been also reported that polysaccharides extracted from *C. sinensis* exhibited many bioactivities, such as antioxidative and anti-tumour activities, and regulating immune functions (Chen, Zhang, Shen, & Wang, 2010; Zhong et al., 2009). For these reasons, great interest centred on finding reliable analytical methods to study the polysaccharides from *C. sinensis*. Significant advances have been made in chemical analysis and bioactivities of the polysaccharides from *C. sinensis* (Leung, Zhao, Ho, & Wu, 2009; Wu, Sun, Qin, Pan, & Sun, 2006; Yu et al., 2006; Zhang et al., 2007; Zhang, Li, Qiu, Chen, & Zheng, 2008). However, conflicting conclusions have been drawn by different research groups about the structure of polysaccharides from *C. sinensis*. Polysaccharides extracted from natural *C. sinensis* had a D-mannose backbone chain with D-galactose branches. However, the backbone of polysaccharides from cultured *C. sinensis* contained mostly mannose and/or glucose (Akaki et al., 2009; Kiho, Ookubo, Usui, Ukai, & Hirano, 1999; Kiho, Tabata, Ukai, & Hara, 1986; Miyazaki, Oikawa, & Yamada, 1977; Wu, Hu, Pan, Zhou, & Zhou, 2007; Wu, Sun, & Pan, 2005; Wu, Ishurd, et al., 2005; Wu et al., 2006; Yalin, Cuirong, & Yuanjiang, 2006). A hydrophilic polysaccharide fraction from cultured *C. sinensis* was found to exhibit antifibrotic effect in renal fibrosis (Zhang, Liu, Al-Assaf, Phillips, & Phillips, submitted for publication). The objectives of the current study were to analyze the monosaccharide composition and elucidate the structure of this bioactive hydrophilic polysaccharide fraction from cultured *C. sinensis* (CBHP) by methylation analysis and 1D and 2D NMR spectroscopy.

2. Materials and methods

2.1. Materials

The bioactive hydrophilic polysaccharide isolated from cultured *C. sinensis* (CBHP) was fractionated at the Glyn O. Phillips Hydrocolloids Research Centre, Glyndwr University as part of a collaboration with the Institute of Nephrology, School of Medicine, Cardiff University and the Zhong Da Hospital, Southeast University, Nanjing, PR China. Fractionation details, molecular characterisation and biological action have been described (Zhang et al., submitted for publication). The weight average molecular weight is M_w 260,000 and contains 0.09% protein.

2.2. Chemical composition of CBHP

Total sugars and uronic acid content were determined using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and meta-hydroxydiphenyl-sulfuric acid method (Blumenkr & Asboehan, 1973), respectively.

Monosaccharide composition of CBHP was determined by hydrolysing samples in 1 M H_2SO_4 at 100 °C for 3 h and diluted, analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The diluted sample were passed through a 0.45 μm filter and injected to HPAEC-PAD system (Dionex-5500, Dionex Corporation, Canada). Separations were achieved with isocratic eluent (100 mM NaOH) on a CarboPac PA1 column (250 mm × 4 mm I.D., Dionex Corporation, Canada) and a guard column (3 mm × 25 mm, Dionex Corporation, Canada) at a flow rate of 1.0 mL/min. The column system was cleaned after each analysis with 200 mM NaOH for 30 min. A post-column delivery system of 600 mM NaOH with a flow-rate of 1.0 mL/min was added to the HPAEC-PAD system. The instrument was controlled and data were processed using Dionex AI 450 software (Dionex Corporation, Canada) (Guo, Cui, Wang, & Young, 2008).

Uronic acid component of CBHP was investigated by an enzymatic-HPLC method (Guo et al., 2008). The sample was dissolved in 0.5 mL 50 mM sodium acetate buffers at room temperature and 0.5 mL of driselase was added. After the mixtures were incubated for 48 h, the mixtures were diluted, and filtered through 0.45 μm filters. Then, the samples were quantitatively analyzed by injected to the HPAEC-PAD with comparison to known standards, galacturonic acid and glucuronic acid. Separations were performed with isocratic eluent (150 mM NaOH) by a CarboPac PA1 column (250 mm × 4 mm I.D., Dionex) at flow rate 1.0 mL/min. The column system was cleaned after each analysis with 500 mM NaOH for 30 min. The instrument was controlled and data were processed using Dionex AI 450 software.

2.3. Methylation and GC–MS for CBHP

Methylation analysis of CBHP was conducted according to the method of Ciucan and Kerek with slight modification (Ciucanu & Kerek, 1984). The dried samples (about 2–3 mg) were dissolved in anhydrous DMSO sonicated at 50 °C for 6.5 h and then heated at 85 °C for 2 h with constant stirring, and then stirred at room temperature overnight to obtain the clear solution. Dry sodium hydroxide (20 mg) was added, and the mixture was stirred for 3 h at room temperature. The mixture was stirred for another 2.5 h after adding 0.3 mL methyl iodide. The methylated sample was then extracted with 1.5 mL methylene chloride. The methylene chloride extract was passed through a sodium sulphate column (0.5 cm × 15 cm) to remove water, and then evaporated by a stream of nitrogen. The dried methylated sample was hydrolysed in 0.5 mL of 4.0 M trifluoroacetic acid (TFA) in a sealed test tube at 100 °C for 6 h and the TFA was removed by evaporation under a stream of nitrogen and dissolved in 0.3 mL distilled water. The hydrolysate was reduced, using 5 mg sodium borodeuteride and acetylated with acetic anhydride (0.5 mL) for 2 h. Borate was removed by repeated additions and evaporations first of 19:1 methanol–acetic acid then methanol alone. The resultant partially methylated alditol acetates (PMAA) were passed through a sodium sulphate column again to remove the water. Aliquots of PMAA were injected on to GC–MS system (ThermoQuest Finnigan, San Diego, CA) fitted with a SP-2330 (Supelco, Bellefonte, PA) column (30 m × 0.25 mm, 0.2 μm film thickness, 160–210 °C at 2 °C/min, and then 210–240 °C at 5 °C/min) equipped with an ion trap MS detector (Guo et al., 2008).

2.4. NMR analysis

228.07 mg CBHP was placed vacuum oven (~80 °C) to dry for about 6 h. Then dried sample was dissolved in 5 mL deuterium oxide (D_2O) (~4%, w/v), then freeze dried. This procedure was repeated three times to completely replace H_2O with D_2O, and sample was finally dissolved in D_2O at room temperature for 3 h before NMR analysis.

Both ^1H and ^{13}C spectra were recorded on a Bruker AMX 500FT spectrometer. The spectra of ^1H, ^{13}C, and homonuclear $^1H/^1H$ correlation experiments (COSY, TOCSY), and Heteronuclear Multiple-Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) Experiments were conducted at 30 °C.

Table 1
Chemical composition of CBHP.

Total sugar (%)	Total uronic acid (%)	Glucose (%)	Mannose (%)	Galactose (%)	Galacturonic acid (%)
96.70 ± 0.35	1.84 ± 0.17	95.19 ± 0.62	0.91 ± 0.03	0.61 ± 0.03	1.32 ± 0.14

3. Results and discussions

3.1. Chemical composition of CBHP

Table 1 shows the chemical composition of CBHP. Although three different monosaccharides and galacturonic acid were found in CBHP, the main constituent was glucose (95.19%). These results indicated that CBHP was a neutral polysaccharide with only a trace amount of uronic acid (1.32%). The high content of glucose would indicate that CBHP has a glucose backbone. Several studies have reported the presence of glucose, mannose and galactose in the polysaccharides from cultured *C. sinensis*, but no uronic acid was

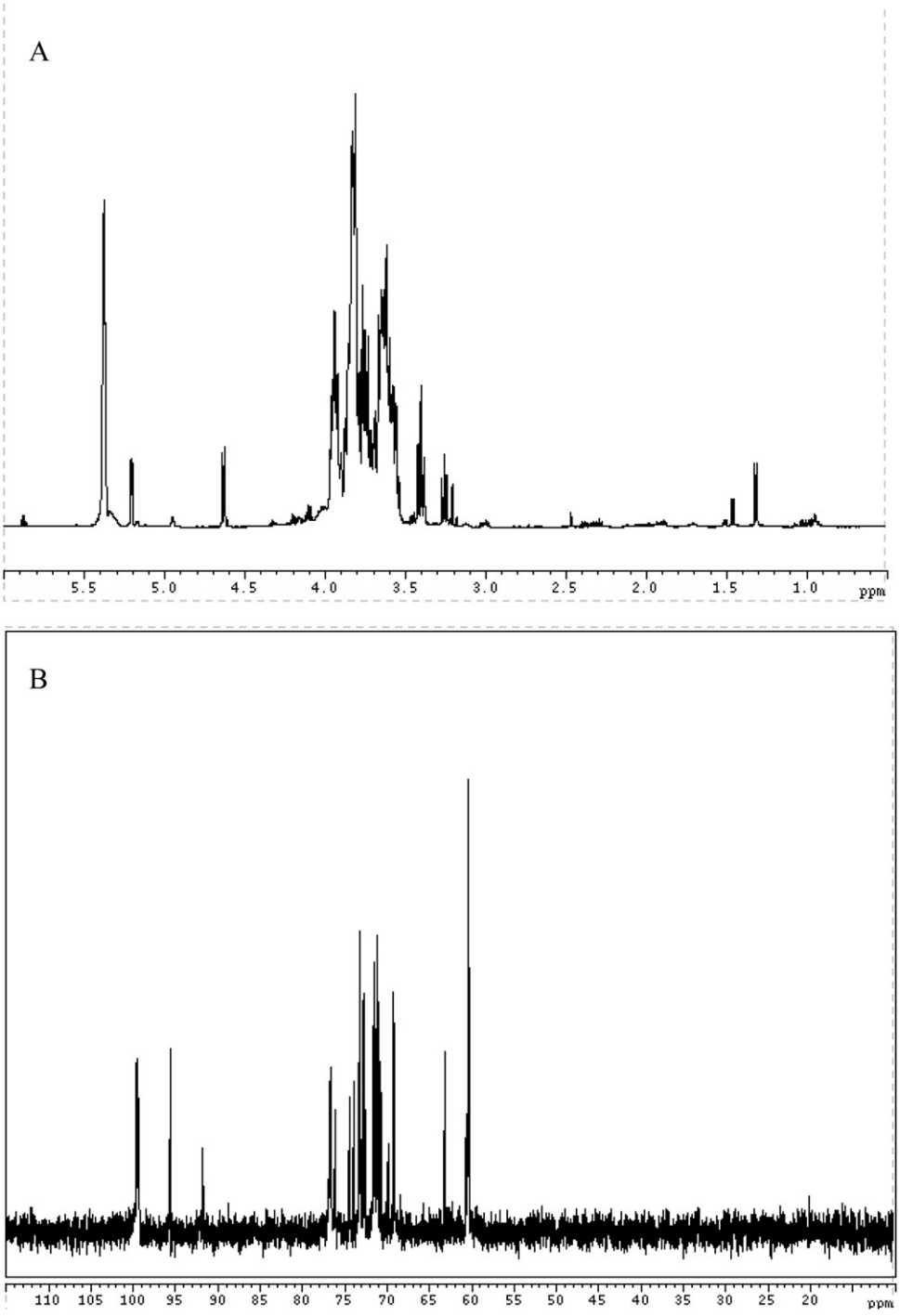

Fig. 1. ^{1}H NMR and ^{13}C NMR spectra of CBHP.

Table 2
Methylation analysis and mode of linkage of CBHP.

Methylated sugar	Deduced linkage	Peak area percentage (%)
1,5-O-Ac_2-2,3,4,6-O-Me_4-glucitol	t-Glcp	20.7
1,2,5-O-Ac_3-3,4,6-O-Me_3-mannitol	1,2-Manp	1.9
1,3,5-O-Ac_3-2,4,6-O-Me_3-galactitol	1,3-Galp	1.0
1,4,5-O-Ac_3-2,3,6-O-Me_3-glucitol	1,4-Glcp	65.7
1,3,5,6-O-Ac_4-2,4-O-Me_2-glucitol	1,3,6-Glcp	2.0
1,4,5,6-O-Ac_4-2,3-O-Me_2 -glucitol	1,4,6-Glcp	1.6
1,2,3,5,6-O-Ac_5-4-O-Me-glucitol	1,2,3,6-Glcp	4.1
1,2,4,5,6-O-Ac_5-3-O-Me-glucitol	1,2,4,6-Glcp	3.0

previously found (Akaki et al., 2009; Kiho et al., 1999). Different extraction methods could account for these small differences.

3.2. Methylation analysis of CBHP

After methylation, the individual peaks of the PMAA and fragmentation patterns were identified by their retention time in GC and by comparison with literature mass spectra patterns (Carpita & Shea, 1989). Based on this analysis of PMAA, the linkage patterns of CBHP are shown in Table 2.

The results indicate that CBHP is a polysaccharide with numerous single sugar unit branch points. The proportion of the non-reducing terminal-D-glucopyranosyl residue is 20.7%. The ratio between apparent terminal units and branching point is 1.16. This is consistent with the fact that the number of polysaccharide branching points approximately equals to the number of terminal units. The data from the methylation analysis suggested that CBHP is mainly composed of a Glc*p* backbone linked 1 → 4 and with some 1 → 3 bonds which could be in the backbone or the side chains. The major branching points of the Glc*p* chain are at O-2 and/or O-6 positions with α- terminal-D-Glc*p* as the side chains. The trace amounts of linkages of 1,2-Man*p* and 1, 3-Gal*p* could only be assigned to incorporated randomly in the side chains. Due to their low amount it is difficult to be precise about their position. Some discrepancies have been reported in the literature. For example, Akaki et al. (2009) assigned the 1 → 3-Glc*p* linkage to the main chain. However, Kiho et al. (1999) claimed that 1 → 5 and or 1 → 6-linked-D-gal*f* should be the backbone. These discrepancies cannot be easily resolved because the samples reported in these studies were from different species and the extraction methods used were also different. The subsequent nuclear magnetic resonance (NMR) spectroscopic analysis confirms the conclusions drawn from methylation analysis and provides us with more details of the structure.

3.3. ^1H NMR, ^{13}C NMR, and 2D NMR analysis

The structural features of CBHP were further elucidated by NMR spectral analysis. The chemical shifts at 5.21 ppm in the ^1H NMR spectrum (Fig. 1A) can be assigned to the typical signals of anomeric protons of α-terminal-D-Glc*p* (A). The high intensity signal at 5.37 ppm was attributed to the anomeric proton of the 1,4-linked-α-D-Glc*p* (B). The two weaker signals with similar relative intensity at 4.64 and 4.62 ppm can be attributed to the anomeric protons of the 1,2,4,6-linked-α-D-Glc*p* (C), 1,2,3,6-linked-α-D-Glc*p* (D), respectively.

The ^{13}C NMR spectrum of CBHP is shown in Fig. 1B. The spectrum exhibited the well resolved signal characteristic of Glc*p*. With respect to anomeric carbons, the chemical shift at 99.51 ppm was identified as α-terminal-D-Glc*p*. The chemical shift at 99.63 ppm is assigned to 1,4-linked-α-D-Glc*p* and 95.67 ppm is assigned to 1,2,4,6-linked-α-D-Glc*p* and 1,2,3,6-linked-α-D-Glc*p*. These values are entirely consistent to literature values (Agrawal, 1992; Ghosh, Chandra, Ojha, & Islam, 2008; Zhao, Li, Luo, & Wu, 2006).

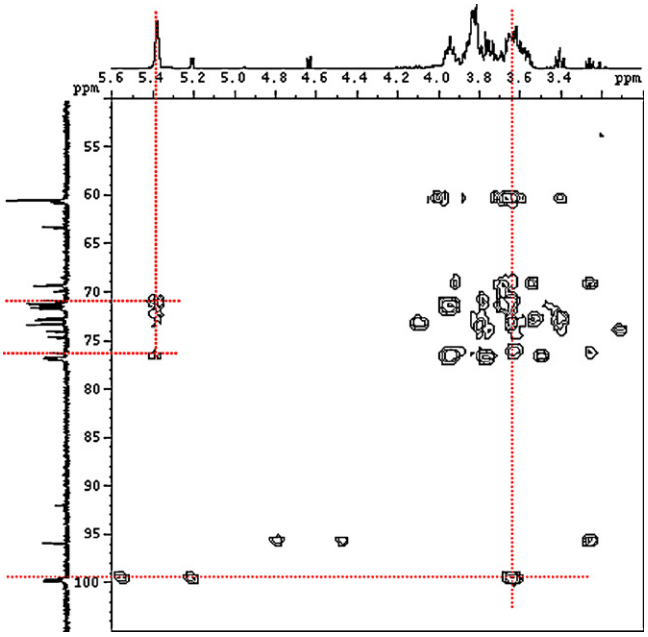

Fig. 2. $^1H/^{13}C$ HMBC correlation of CBHP.

The HMQC spectrum (spectrum not presented) showed the spectrum of distinct cross peaks in the anomeric region of Glc*p*. The C-1 signals at 99.51, 99.63, 95.67, 95.67 ppm can be assigned to the α-terminal-D-Glc*p*, 1,4-linked-α-D-Glc*p*, 1,2,4,6-linked-α-D-Glc*p* and 1,2,3,6-linked-α-D-Glc*p*, respectively. These signals cross link to the proton signals at chemical shifts 5.21, 5.37, 4.64 and 4.62 ppm, respectively. The HMBC spectrum (Fig. 2) indicated that the H-1 signals of 1,4-linked-α-D-Glc*p* correlate with the C-4 of 1,4-linked-α-D-Glc*p*, and 1,2,4,6-linked-α-D-Glc*p* residues. It was also observed that H-1 of 1,4-linked-α-D-Glc*p* correlates with the C-3 of 1,2,3,6-linked-α-D-Glc*p*. Reversely, the cross peak of C-1 of 1,4-linked-α-D-Glc*p* correlates with the H-4 of 1,4-linked-α-D-Glc*p* (Fig. 2). COSY (Fig. 3) enables us to assign the chemical shifts of the anomeric protons from the α-terminal-D-Glc*p*, 1,4-linked-α-D-

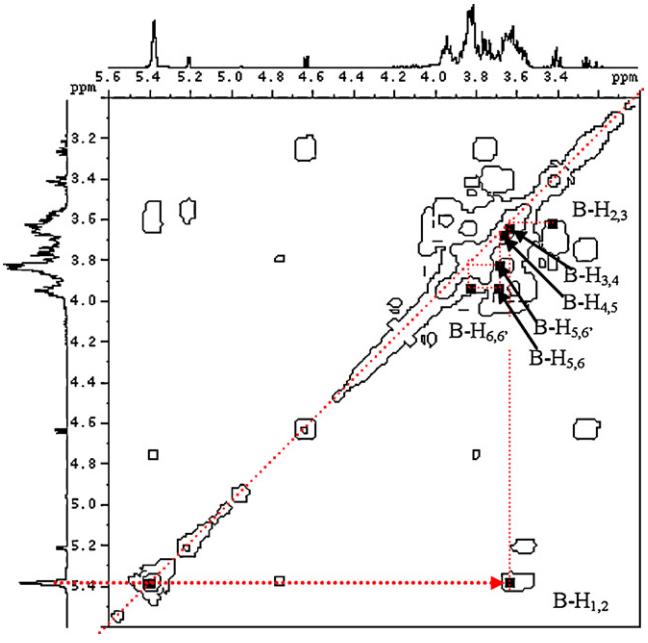

Fig. 3. $^1H/^1H$ COSY correlation of CBHP.

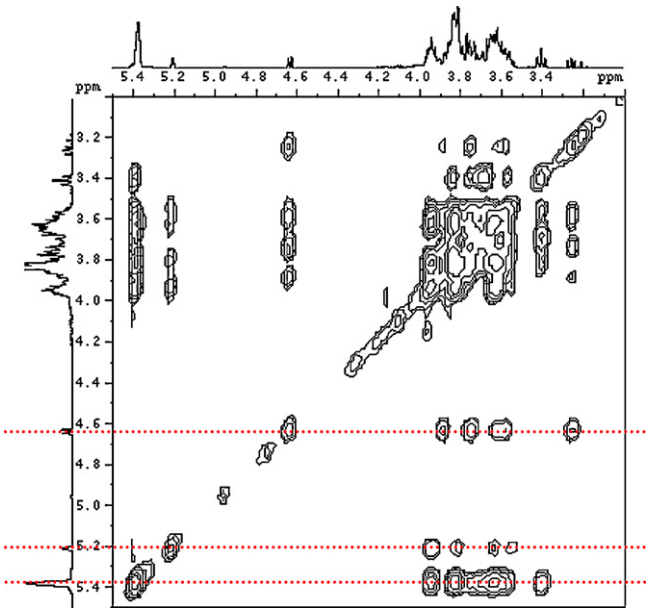

Fig. 4. $^{1}H/^{1}H$ TOCSY correlation of CBHP.

Table 3
Chemical shift (δ) assignments of ^{1}H NMR and ^{13}C NMR spectra of CBHP on the basis of HMQC, HMBC, COSY and TOCSY.

Glucosyl residues	Assigned H, C position	^{1}H (ppm)	^{13}C (ppm)
A: α-D-Glcp-(1→	1	5.21	99.51
	2	3.57	71.20
	3	3.92	73.10
	4	3.78	70.73
	5	3.82	72.61
	6	3.66, 3.95	63.12
B: 1,4-Linked-α-D-Glc	1	5.37	99.63
	2	3.62	71.08
	3	3.41	69.84
	4	3.62	71.63
	5	3.64	71.45
	6	3.81, 3.94	60.38
C: 1,2,4,6-Linked-α-D-Glc	1	4.64	95.67
	2	3.25	73.9
	3	3.75	73.22
	4	3.90	72.66
	5	3.75	76.08
	6	3.65, 3.90	74.63
D: 1,2,3,6-Linked-α-D-Glc	1	4.62	95.67
	2	3.23	74.4
	3	3.74	72.76
	4	3.88	76.61
	5	3.74	73.32
	6	3.62, 3.88	76.59

Glc*p*, 1,2,4,6-linked-α-D-Glc*p* and 1,2,3,6-linked-α-D-Glc*p* coupling with their respective H-2s. The TOCSY spectrum provided all intra-residue connectivity of all protons of the α-terminal-D-Glc*p* and 1,4-linked-α-D-Glc*p*, 1,2,4,6-linked-α-D-Glc*p*, 1,2,3,6-linked-α-D-Glc*p*, respectively, as shown in Fig. 4. Thus when we combine the information from the ^{1}H and ^{13}C NMR, HMQC, HMBC, COSY, and TOCSY spectroscopy, a complete assignment of all the linkage patterns can be identified as shown in Table 3.

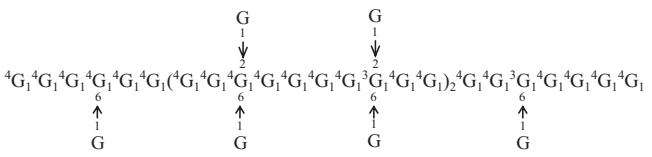

Fig. 5. The proposed structure of CBHP.

4. Conclusion

Summarizing the information from methylation analysis, 1D and 2D NMR spectroscopy, we conclude that structure of CBHP comprises a backbone of Glc*p* α-linked 1 → 4 with 1 → 3 linked branches at O-2 or O-6 of Glc*p* with an α- terminal-D-Glc*p* in the side chain. The trace amount of linkages of 1,2-Man*p* and 1, 3-Gal*p* probably occur randomly in the side chains. The schematic proposed structure of CBHP is shown in Fig. 5.

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