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Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*

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ABSTRACT

A novel polysaccharide named CBP-1 was isolated from the fruiting body of cultured *Cordyceps militaris* by alkaline extraction as well as anion-exchange and gel-permeation chromatography. Its structural features were investigated by a combination of chemical and instrumental analysis approaches, including partial hydrolysis, methylation analysis, HIO_4 oxidation-Smith degradation, GC–MS, ^{13}C NMR, HPAEC-PAD and FT-IR. The results indicated that CBP-1 has a backbone of (1 → 4)-α-D-mannose residues which occasionally branches at O-3. The branches were mainly composed of (1 → 4)-α-D-glucose residues and (1 → 6)-β-D-galactose residues, and terminated with β-D-galactose residues. In the *in vitro* antioxidant assay, CBP-1 was found to possess the hydroxyl radical-scavenging activity with an IC_{50} value of 0.638 mg/ml.

1. Introduction

The particular interest in the structure of fungal extracellular polymers has noticeably increased since the discovery of their several physiological roles in fungi morphogenesis, in their association with hosts and by their mobilization as food reserve (Kremar, Novotny, Marais, Joseleau, 1999). Many natural polysaccharides and polysaccharide–protein complexes have been isolated from fungi and used as a source of therapeutic agents (Methacanon, Madla, Kirtikara, & Prasitsil, 2005; Yu, Song, et al., 2004).

Cordyceps militaris is also widely known as the Chinese rare caterpillar fungus, and has similar, if not superior, pharmacological activities to the famous Chinese traditional medicine *C. sinensis* in the treatment of certain diseases (Gai, Jin, Wang, Li, & Li, 2004; Zheng & Cai, 2004; Zhu, Halpern, & Jones, 1998). It is commonly used to replenish the kidney and soothe the lung for the treatment of hyposexualtiy, hyperglycemia, hyperlipidemia, renal dysfunction and liver disease in traditional Chinese medicine (Won & Park, 2005; Yu, Wang, Zhang, Zhou, & Zhao, 2004). Recently several studies have demonstrated that the extracts of *C. militaris* have multiple pharmacological actions such as anti-inflammation Won & Park (2005), improvement of insulin resistance and insulin secretion, and antioxidant activity stronger than that of *C. sinensis* and *C. kyushuensis* (Yu et al., 2007). Due to rarity and consequent high cost of the natural *C. militaris*, many scientists have extensively examined its life cycle with the aim of developing techniques for isolating fermentable strains. Several strains have been isolated from natural *C. militaris* and manufactured in high quality by fungus-cultivation technology. The products from cultured *C. militaris* have shown similar pharmacological efficacy comparable to that of natural *C. militaris* (Wang & Zhong, 2002). Cultivated fruiting bodies of *C. militaris* were commonly sold as drug materials and health food products in China and South East Asia (Li, Yang, & Tsim, 2006).

In the last few years, the structures of several polysaccharides isolated from *Cordyceps spp* were reported (Methacanon et al., 2005; Wu, Sun, & Pan, 2006; Xiao et al., 2006; Yu, Song, et al., 2004; Yu, Wang, et al., 2004). The structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *C. militaris* was reported recently by our research group (Yu et al., 2007). The water-soluble crude polysaccharides were obtained from the fruiting bodies of cultured *C. militaris* using hot water extraction followed by ethanol precipitation. The polysaccharides were successively purified by chromatography on DEAE–cellulose-52 and Sephacryl S-100 HR columns. Structural features of P70-1 were investigated by a combination of chemical and instrumental analysis. In the *in vitro* antioxidant assay, P70-1

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was found to possess hydroxyl radical-scavenging activity with an IC_{50} value of 0.548 mg/ml. In this paper, the polysaccharides were further fractionated and several distinctive alkali-extract polysaccharides were obtained. Among them, CBP-1 exhibited inhibitory activity towards hydroxyl radicals with an IC_{50} value of 0.638 mg/ml. Therefore, the aim of this study was to investigate the complete structure of CBP-1.

2. Experimental

2.1. Material

Cultured *C. militaris* was obtained from Shenyang Zhongtian Bioengineering Corporation, Shenyang, China. The material (No. 05-09-0001) was identified by Professor R.M. Yu, College of Pharmacy, Jinan University, China.

Ascorbic acid (Vitamin C, Vc), hydrogen peroxide (H_2O_2), ferrous sulfate ($FeSO_4$), and brilliant green were purchased from Shanghai Chemical Reagent Company, Shanghai, China. Standard dextrans T−500 (molecular weight: 500 KDa), T−70 (molecular weight: 70 KDa), T−40 (molecular weight: 40 KDa) and T−10 (molecular weight: 10 KDa) were purchased from Pharmacia. All other reagents were the products of Sigma Chemical Co. All reagents were of analytical grade.

2.2. General methods

The total sugar content of CBP-1 was determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Optical rotations were measured with a Jasco P-1020 polarimeter. IR spectra were recorded with a Tensor 27 Bruker instrument with KBr pellets. ^{13}C NMR Spectra were recorded with a Bruker 500 instrument, and the sample was dissolved in D_2O. High performance anion exchange chromatography (HPAEC) was analyzed on a Dionex ICS-2500 system, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC ^{TM}PA10 (2.0 × 250 mm) column. GC was analyzed on an Agilent 190911 J-413 HP-5 equipped with FID, inositol as an internal standard; GC–MS was conducted with a Hewlett Packard 5895 instrument, using a fused-silica capillary column (30 × 25 mm) coated with a 0.2 mm film of DB-5. The ionisation potential was 70 eV and the temperature of the ion source was 220 °C.

2.3. Extraction and fractionation of polysaccharides

Three hundred grams of the dried powder of cultured *C. militaris* was defatted with ethanol for 10 h and extracted three times with hot water (50 °C), each time for 10 h. Then further extraction was conducted with 0.3 mol/L NaOH. This alkali extract was concentrated to a volume of 100 ml under reduced pressure. The crude polysaccharide fraction, termed CBP, was obtained by ethanol precipitation at the final concentrations of 80% of ethanol.

2.4. Isolation and purification of the polysaccharide

Sevag reagent (1-butanol/chloroform, v/v = 1:4) (Staub, 1965) was used for the deproteination of CBP, 30% H_2O_2 for decoloration, respectively. CBP was dialyzed against tap water and distilled water for 48 h. The resulting polysaccharide solution was concentrated and lyophilized. Ion-change chromatography and gel filtration column chromatography were used for the isolation of these preparations. Each sample (400 mg) was dissolved in 0.025 M Tris–HCl (pH 7.0), centrifuged, and then the supernatant was injected to a column of DEAE-Cellulose-52 equilibrated with 0.025 M Tris–HCl (pH 7.0). After loading with sample, the column was eluted with gradient NaCl aqueous solution (0–1 M), and the procedure was monitored by the phenol-sulfuric acid method mentioned above. The fractions were further purified by gel filtration chromatography on a column of Sephacryl S-100 HR. The polysaccharide CBP-1, which had $[\alpha]_D^{20}$+ 158° (c0.5, water), was obtained by the above processes and used for structural elucidation and bioactivity determination.

2.5. Measurement of molecular weight of CBP-1

Gel chromatographic method (Wang, Liang, & Zhang, 2001) was used for determination of molecular weight of CBP-1. Standard dextrans T−500, T−70, T−40, and T−10 were passed through a Sephacryl S−300 HR column, and elution volumes were plotted against the logarithms of their respective molecular weights. A solution of the polysaccharide (5 mg) in distilled water (0.5 ml) was applied to the column equilibrated and eluted with distilled water at a fixed flow rate (10 ml/h). Elution volume of the polysaccharide was then plotted in the same graph, and molecular weight of CBP-1 was measured.

2.6. Analysis of monosaccharide composition of CBP-1

Ten milligrams of CBP-1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C in a sealed-tube for 8 h. Excess acid was removed by co-distillation with MeOH after the hydrolysis was completed. One part of the hydrolysate was analyzed by GC, and the other was measured by HPAEC−PAD.

GC analysis: The hydrolysate and inositol were reduced by $NaBH_4$, followed by acidification with acetic acid. Then co-distillation with MeOH was performed in order to remove excess boric acid and dried over P_2O_5. Thereafter, the sugars were treated with pyridine (2 ml) and acetic anhydride (1.5 ml) to convert into their alditol acetate and analyzed by GC at a temperature program of 50–230 °C with a rate of 2 °C/min.

HPAEC−PAD analysis: The hydrolysate (1 mg) was dissolved in pure water (1 mg/ml).

Twenty-five microliters of this solution was used for the ionic-chromatography analysis by HAPEC−PAD of Dionex ICS-2500 System, eluted with a mixture of water and 200 mM NaOH in the volume ratio of 92:8.

2.7. Partial acid hydrolysis of CBP-1

One hundred milligrams of CBP-1 were hydrolyzed with 0.05 M TFA for 6 h at 100 °C, centrifuged, dialyzed with distilled water for 48 h, and then diluted in the dialysis sack with ethanol. The precipitate (A) and supernatant (B) in the sack and the fraction out of sack (C) were obtained after hydrolysis, and three fractions were dried and analyzed with GC.

2.8. Periodate oxidation-Smith degradation reaction of CBP-1

CBP-1 (20 mg) was allowed to swell overnight in distilled water (10 ml), dispersed using a blender, and upon addition of 15 mM $NaIO_4$ (25 ml), an immediate reduction in viscosity occurred. The solution was kept in the dark at 4 °C, 30 μl aliquots were withdrawn at 6 h intervals, diluted to 5 ml with distilled water and read in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001). Complete oxidation, identified with a stable absorbance, was reached in 96 h. Consumption of HIO_4 was measured by a spectrophotometric method (Aspinall & Ferrier, 1957), and formic acid production was determined by titration with 0.053 M NaOH. Two milliliters of glycol was added, and then the experiment of periodate oxidation was over.

The rest of the periodate product was exhaustively dialyzed against tap water (48 h) and distilled water (24 h), respectively. The nondialysate was concentrated and reduced with sodium borohydride (40 mg) for 24 h at room temperature, and then the pH of the solution was adjusted to 5.0 by adding 0.1 M acetic acid, dialyzed against distilled water (24 h) and the nondialysate was dried in vacuum. The product obtained was hydrolyzed with 2 M TFA (3 ml) at 100 °C for 6 h. The components of this polyalcohol as the alditol acetate were analyzed by GC under the same conditions as those used for the experiment of monosaccharide composition analysis (Zhao, Kan, Li, & Chen, 2005).

2.9. Methylation analysis of CBP-1

Ten milligrams of CBP-1 was used in this analysis according to the method described by Hakomori (Hakomori, 1964). The methylated polysaccharide was treated with 90% aqueous formic acid (3 ml) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M TFA (2 ml) under the above conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride, and analyzed as the alditol acetates by GC–MS. The identification of the methylated sugars linkages were identified on the basis of relative retention time and fragmentation pattern (Needs & Selvendran, 1993). Molar ratios for each sugar were calibrated using the peak areas and response factor of the flame-ionisation detector in GC.

2.10. Determination of hydroxyl radical-scavenging activity of CBP-1

The hydroxyl radical system generated by the Fenton reaction was used for the evaluation of *in vitro* antioxidant activity of CBP-1 (He, Luo, Cao, & Cui, 2004). Briefly, the reaction mixture contained 1.0 ml of brilliant green (0.435 mM), 2.0 ml of $FeSO_4$ (0.5 mM), 1.5 ml of H_2O_2 (3.0%) and samples of various concentrations. After incubation at room temperature for 20 min, the absorbance of the mixture was measured at 624 nm. Hydroxyl radicals bleached the brilliant green, so the absorbance change of the reaction mixture indicated the scavenging ability for hydroxyl radicals. The hydroxyl radical-scavenging activity was expressed as:

$$\text{Scavenging rate } (\%) = [(A_s - A_0)]/[(A - A_0)] \times 100\%$$

where A_s is the absorbance in the presence of the sample, A_0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and Fenton reaction system.

2.11. Statistical analysis

Tests were carried out in triplicate for three separate experiments. Values are presented as means ± SD. The IC_{50} value is the amount of sample needed to inhibit free radicals concentration by 50%, which can be calculated from the concentration–response curves (SigmaPlot 2001 software).

3. Results and discussion

3.1. Isolation, purification and composition of polysaccharides

The crude polysaccharide was obtained from the alkaline extraction of cultured *C. militaris* with a yield of 6%. After fractionation on DEAE-Cellulose 52 and Sephacryl S-100HR column, 908 mg of CBP-1 was obtained from the NaCl eluate. The homogeneity of the polysaccharide was elucidated by the following tests. It was eluted as a single peak from gel-filtration chromatography on Sephacryl S-300 HR column and had the same optical rotation in different low content aqueous ethanol by Jasco P-1020 automatic optical polarimeter at room temperature. Average molecular weight, total sugar content, specific rotations, and monosaccharide compositions of the polysaccharide were determined and shown in Table 1.

Table 1
Components of monosaccharide and properties of CBP-1 from cultured *C. militaris*

Properties	Data	
$[\alpha]_D^{20}$	+152.0	
Molecular weight (kDa)	17	
Carbohydrate (wt %)	99.9%	
Monosaccharide component (mol)		
Man	2.81[a]	3.15[b]
Gal	4.01[a]	4.34[b]
Glc	1.00[a]	1.00[b]

[a] Analyzed by HPAEC-PAD.
[b] Analyzed by GLC.

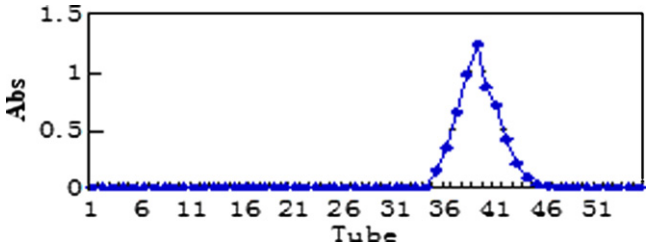

Fig. 1. Profile of CBP-1 in Sephacryl S-300 HR column chromatography.

A single elution peak appeared for CBP-1 in Sephacryl S-300 HR (Fig. 1) and was detected by the phenol-sulfuric acid assay. The alditol acetate of CBP-1's hydrolysate was measured by GC. HPAEC-PAD chromatogram profiles of standard monosaccharide mixture solution and hydrolysate of CBP-1 were shown in Fig. 2. The three monosaccharides, D-Man, D-Gal and D-Glc, were identified in the hydrolysate of CBP-1, and their ratios were 2.81:4.01:1.00 by HPAEC-PAD and 3.15:4.34:1.00 by GC–MS.

3.2. Structural elucidation of CBP-1

CBP-1 was supposed to be the D-configuration according to the determination of optical rotation, ^{13}C NMR data and GC analysis. No absorption at 280 and 260 nm in UV absorption spectra of CBP-1 appeared and demonstrated possible absence of protein and nucleic acid in this polysaccharide.

IR spectra of CBP-1 showed absorption bands at 3398, 2931, 2130, 1647, 891 and 840 cm^{-1}. The absorption bands at 840 and 891 cm^{-1} indicated that CBP-1 contained both α- and β-type glycosidic linkages in its structure.

Three fractions were obtained after partial acid hydrolysis of CBP-1. The alditol acetates of each fraction hydrolysate were subjected to GC analysis, and the results are shown in Table 2.

Fraction A, the material left in the sack after dialyzing of the partial acid hydrolyzate, possesses the biggest size among three fractions. The component of fraction A, in which the ratio of mannose:glucose:galactose was 4.230:1:2.822, indicating that mannose may be the backbone of the structure of CBP-1, and glucose may be close to this backbone. The analysis results of fraction B and fraction C indicated that the branched structure of CBP-1 was mainly composed of galactose.

Fig. 3 showed the result of methylated CBP-1 on GC–MS. Methylation analysis of fractions CBP-1 showed the presence of five components (Table 3), namely 2, 3, 4, 6-Me_4-Gal, 2, 3, 6-Me_3-Man, 2, 3, 6-Me_3-Glc, 2, 3, 4-Me_3-Gal, 2, 6-Me_2-Man in molar ratio of 1.23:2.25:1.31:3.42:1.16 (about 1:2:1:3:1). This showed a good correlation between terminal and branched residues. In addition,

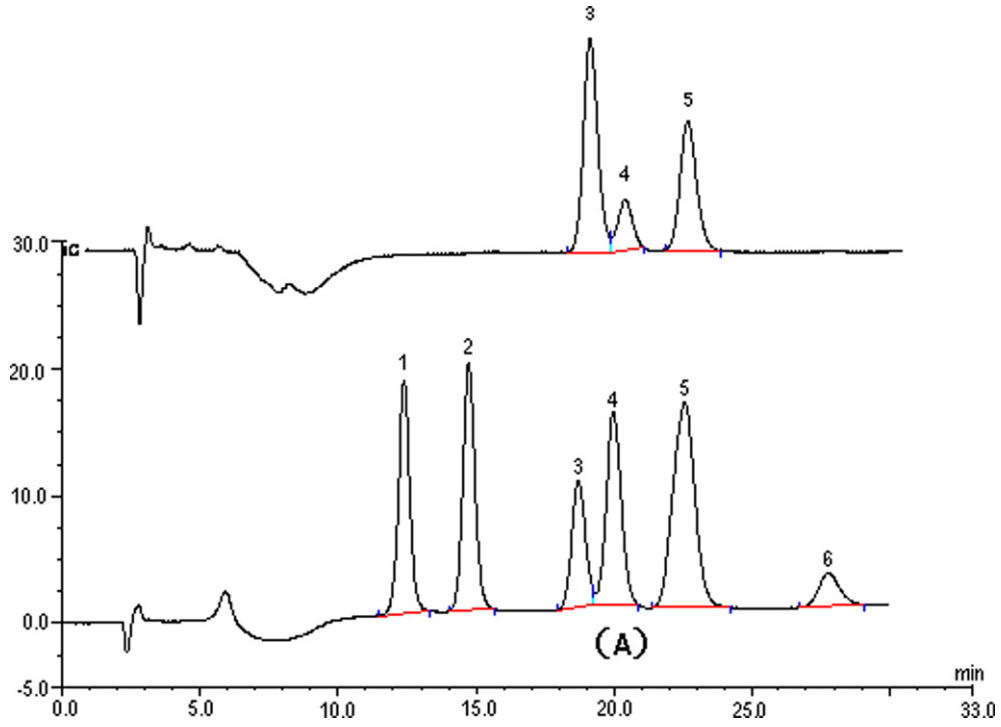

Fig. 2. HPAEC-PAD chromatogram profile of standard monosaccharide mixture solution (A) and hydrolysate of CBP-1 (B). (A) Peak identy: (1) L-Rha (rt: 12.384); (2) L-Ara (rt: 14.717); (3) D-Gal (rt: 18.684); (4) Glc (rt: 19.950); (5) D-Man (rt: 22.534); (6) D-Fru (rt: 27.800). (B) Peak identity: (3) D-Gal (rt: 18.634); (4) D-Glc (rt: 19.884); (5) D-Man (rt: 22.167).

Table 2
Results of GC analysis for partial acid hydrolysis of CBP-1

Fractions	Molar ratios		
	D-mannose	D-glucose	D-galactose
A	4.230	1	2.822
B	2.501	1	2.477
C	0.456	1	6.363

A, precipitation in the sack.
B, supernatant in the sack.
C, fraction out of the sack.

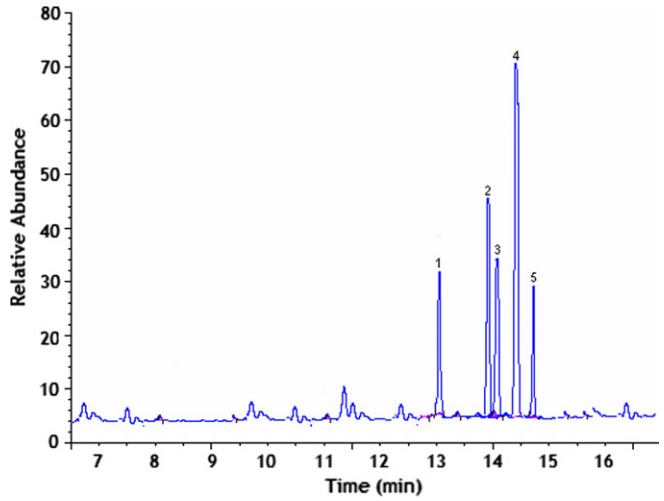

Fig. 3. GC profile of methylated CBP-1 on GC–MS. Peak identy: (1) 2,3,4,6-Me_4-Gal (rt: 13.01); (2) 2,3,6,-Me_3-Man (rt: 13.95); (3) 2,3,6-Me_3-Glc (rt: 14.09); (4) 2,3,4-Me_3-Gal (rt: 14.45); (5) 2, 6-Me_2-Man (rt: 14.79).

these molar ratios also agree with the monosaccharide composition of CBP-1 described above.

The periodate-oxidated product of CBP-1 was hydrolyzed and tested by HPAEC-PAD. Mannose, glycerin and erythritol, were found on the GC spectrum. The presence of mannose, indicating a part of mannose is in 1, 3-, 1, 2, 3-, 1, 2, 4-, 1, 3, 4-, 1, 3, 6-, or 1, 2, 3, 4-linkage, which cannot be oxidized by HIO_4. Because of the absent of galactose and glucose in the hydrolyzed product, we may suppose that galactose and glucose are in linkages that can be oxidized, namely 1-, 1, 6-, 1, 2-, 1, 2, 6-, 1, 4-, or 1, 4, 6-linkage. Based on the result from methylation analysis, it could be concluded that mannose was derived from 1, 3, 4-linked mannose residues, glycerin from 1, 3, 4-linked mannose and 1-linked galactose, and erythritol from 1, 4-linked glucose and 1, 4-linked mannose.

HIO_4 consumption and formic acid production of the polysaccharide were 1.361 mol/mol sugar residue and 0.504 mol/mol sugar residue, respectively, on periodate oxidation, which was also in agreement with the theoretically calculated values based on the structural features described above.

According to the literature (Wang, Luo, & Liang, 2004; Wang et al., 2001; Zhang, 1999; Zhao et al., 2005), the resonances in the region of 98–106 ppm in the ^{13}C NMR spectrum of CBP-1 (Fig. 4) were attributed to the anomeric carbon atoms of mannopyranose (Man*p*), galactopyranose (Gal*p*) and glucopyranose (Glc*p*). The peak at 103.3 ppm corresponded to C-1 of β-D-galactopyranose (Gal*p*) residues. The assignment of the carbon atoms signals was shown in Table 4.

Both results of partial acid hydrolysis and methylated linkage analysis of CBP-1 indicated that (1 → 4)-linked-mannose was one of the largest amounts residue of the polysaccharide structure, the branched residue was (1 → 3, 4)-linked mannose revealing that (1 → 4)-linked-mannose should be possible to form the backbone structure. The relative amounts of (1 → 3, 4)-linked-mannose indicating that approximate branch ratios could theoretically be 33%,

Table 3
GC–MS results of methylation analysis of CBP-1

Methylated sugar	Ret. time (min)	Molar ratios	Mass fragments (m/z) (Relative abundance, %)	Linkage type
2, 3, 4, 6-Me_4-Gal	13.01	1.23	45, 87, 101, 117, 145, 161, 205 (100, 43, 11, 8, 3, 1.8, 1.2)	T-
2, 3, 6, -Me_3-Man	13.95	2.25	45, 87, 101, 113, 117, 161, 233 (100, 36, 23, 14, 18, 5, 2.3)	1, 4-
2, 3, 6-Me_3-Glc	14.09	1.31	45, 87, 101, 113, 117, 161, 233 (100, 38, 21, 15, 19, 7, 2.4)	1, 4-
2, 3, 4-Me_3-Gal	14.45	3.42	45, 87, 101, 189, 233 (100, 29, 16, 4.3 1.9)	1, 6-
2, 6-Me_2-Man	14.79	1.18	71, 87, 99, 101, 189 (100, 63, 35, 14, 4.7)	1, 3, 4-

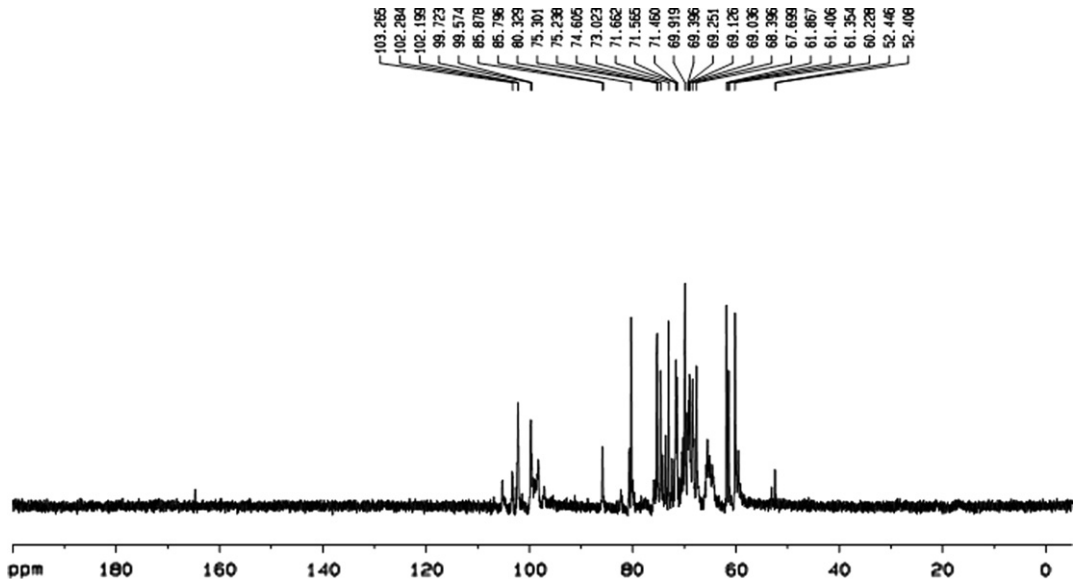

Fig. 4. The ^{13}C NMR spectrum of CBP-1.

Table 4
Assignment of ^{13}C NMR chemical shift values of CBP-1

Sugar residues	Chemical shifts, δ (ppm)					
	C1	C2	C3	C4	C5	C6
→ 4)-α-D-Glc*p*(1 →	99.723	69.396	73.023	80.329	71.460	60.228
→ 4)-α-D-Man*p*(1 →	102.284	69.036	73.023	80.329	75.301	61.867
→ 6)-β-D-Gal*p*(1 →	99.574	69.126	71.565	71.460	73.023	68.396
β-D-Gal*p*(1 →	103.265	69.251	75.238	71.565	74.605	61.406
→ 4)-α-D-Man*p*(1 → (3←	102.199	69.719	85.878	85.796	75.301	67.699

namely on average one branching point for each three residues of backbone. Residues of branch structure were (1 → 4)-linked-glucose and (1 → 6)-linked-galactose, which is in agreement with those data of partial acid hydrolysis and Smith degradation.

It is concluded that a repeating unit of CBP-1 has a backbone of (1 → 4)-α-D-mannose residues which branches at O-3 based on the experimental results. The branch was supposed to be the composition of an (1 → 4)-α-D-glucose residue and three (1 → 6)-β-D-galactose residues, and terminated with β-D-galactose residues. The predicted structure of the novel polysaccharide CBP-1 was shown in Fig. 5.

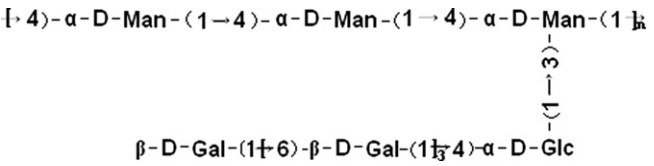

Fig. 5. Predicted structure of CBP-1.

3.3. Hydroxyl radical-scavenging activity of CBP-1

The results of hydroxyl radical-scavenging activities of CBP-1 and Vc used as a positive control (He et al., 2004) were given in Fig. 6. As is illustrated in the figure, CBP-1 exhibited the scavenging activity towards hydroxyl radicals in a concentration-dependent manner, with an IC_{50} value of 0.638 mg/ml. Vc showed a slightly stronger effect on the hydroxyl radicals, with an IC_{50} value of 0.370 mg/ml, under the same conditions.

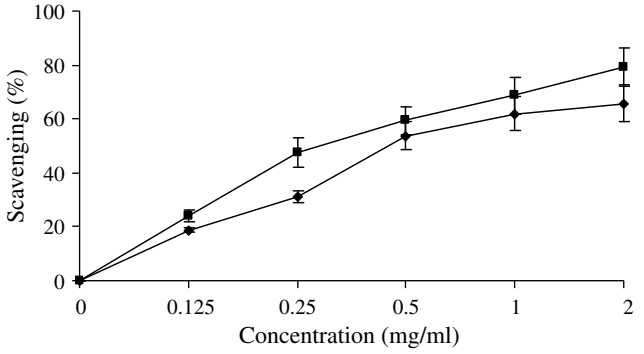

Fig. 6. Hydroxyl radical-scavenging activity of CBP-1 and Vc. Values are means ± SD of three determinations. Symbols: ♦, CBP-1; ■, V_c.

Hydroxyl radicals, superoxide anions and hydrogen peroxides as reactive oxygen species (ROS), are related to the pathogenesis of various diseases. Hydroxyl radical is the most reactive among the oxygen radicals and induces severe damage to the adjacent biomolecules. The oxidative injury caused by hydrogen peroxide and superoxide molecules is also associated with the indirect pro-

duction of hydroxyl radicals via Fenton reaction and iron-catalyzed Haber–Weiss reaction. Given the above knowledge, the Fenton reaction system was logically chosen in the present study for the evaluation of CBP-1's *in vitro* antioxidant activity.

Some polysaccharides from plants, animals and fungi hold much promise as antioxidative compounds. The afore-mentioned polysaccharides are one of the main bioactive components in *Cordyceps* spp. Our findings indicate that CBP-1 is capable of scavenging hydroxyl radicals, which provides important experimental evidence for supporting potential clinical applications of *C. militaris* as a substitute for *C. sinensis* in Chinese traditional medicines. To best understand the bioactivity of CBP-1, further studies on the mechanism of its scavenging activity against hydroxyl radicals is currently underway.

4. Conclusion

The results reported in this paper demonstrate that the novel polysaccharide obtained by alkaline extraction from cultured *C. militaris* is a heteropolysaccharide and is slightly branched. Antioxidation tests suggest that CBP-1 possesses the hydroxyl radical-scavenging activity with an IC_{50} value of 0.638 mg/ml. Further work is necessary to reveal detailed pharmacological effects of CBP-1 and other secreted substances that could co-exist in alkali-extracted crude polysaccharide. The pharmacological results obtained in this study will substantially aid in elucidating the use of cultured *C. militaris* roots in Chinese traditional medicine.

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