Carbohydrate Polymers 75 (2009) 166–171

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 31 December 2007
Received in revised form 4 May 2008
Accepted 11 July 2008
Available online 22 July 2008

Keywords:
Cultured *Cordyceps militaris*
Fruiting bodies
Alkaline extraction
Polysaccharide structure
HPAEC-PAD analysis
Antioxidant

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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doi:10.1016/j.carbpol.2008.07.023

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.