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Full Length Research Paper

Structural characterization and antioxidant activity of purified polysaccharide from cultured *Cordyceps militaris*

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The polysaccharide fraction, CM-hs-CPS2, was isolated from fruiting bodies of cultured *Cordyceps militaris* grown on solid rice medium by hot water extraction and ethanol precipitation, and purified by ion exchange column (DEAE-cellulose-52) and gel filtration column (Sephadex G-100) chromatography. Its structural characterizations were analyzed by gas chromatograph (GC) and fourier transform infrared spectroscopy (FT-IR). Results indicated that CM-hs-CPS2 was a kind of sulfating acidic polysaccharide containing acetamido group. The monosaccharide composition analysis showed that CM-hs-CPS2 was composed mainly of mannose, glucose and galactose in a molar ration of 1.35: 8.34: 1.00, and linked by α-glycosidic linkage. The studies on antioxidant activities of CM-hs-CPS2 were evaluated by various methods *in vitro*. Results showed that DPPH· scavenging activity, reducing power and ferrous ion chelating activity of CM-hs-CPS2 (8 mg/ml) were 89%, 1.188 and 85%, respectively.

Key words: *Cordyceps militaris*, polysaccharide, structure, bioactivity.

INTRODUCTION

Cordyceps sinensis Berk. Sacc, as a tonic herb in Chinese traditional medicine, is one of entomogenous fungi that form a fruiting body mainly on an insect larva. It has been widely used as a general tonic for protecting and improving lung and kidney functions (Leung et al., 2009), anti-cancer (Bok et al., 1999), immunomodulation (Kuo et al., 2001) and hypotensive effect (Chiou et al., 2000).

However, naturally occurring *C. militaris* is not easily available for food or medicine in large amounts because of its rare and expensive. Therefore, much effort has been invested to look for alternative species. *C. militaris*, a species of *Cordyceps* spp., has also been used as a folk medicine in China for hundred years. Studies have proved that it possesses similar constituents and pharmacological efficacy to those of *C. sinensis* (Wei et al., 2004; Zheng et al., 2004). As a result, *C. militaris* is considered to be the suitable alternative of *C. sinensis* (Gui et al., 2008).

Polysaccharide is one of major bioactive constituents of *Cordyceps* and many other medicinal fungi, which has proven anti-cancer, immunomodulation activities (Wasser, 2002), and the notable anti-oxidation effect (Liu et al., 1997). The anti-oxidant properties of herbs are most relevant to their health protecting functions.

The anti-oxidant function of polysaccharides from various natural products has been extensively studied. However, studies on the anti-oxidant properties of *C. militaris* polysaccharides are seldom reported. The aim of this study was to better understand and characterize the structural characteristics of the polysaccharide, CM-hs-CPS2, which was isolated and purified from the strain CM-hs of cultured *C. militaris* in our lab. Furthermore, we investigated antioxidant activity of CM-hs-CPS2 *in vitro*. The results will be helpful for further revealing the

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relationship between structure and bioactivities of *C. militaris* polysaccharides.

MATERIALS AND METHODS

Fungal strains and Materials

The *Cordyceps militaris* strain, CM-hs, was conserved in in the lab of biological resource function, Jiangsu University of Science and Technology and used for this study. Fresh fruiting bodies grown on solid rice medium were obtained. Ascorbic acid (Vitamin C), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, potassium ferricyanide, ferrous chloride and ferric chloride were purchased from Bio Basic Inc of China. Other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. All reagents were of analytical grade.

Extraction of polysaccharides

The dried powder of cultured *C. militaris* (100 g) was defatted with ethanol at 70℃ for 10 h at twice, and exhaustively extracted with 20 volumes of water at 80℃ for 10 h at twice. The extract was concentrated under reduced pressure to a volume of 100 ml, deproteinated by Sevag method (Liu et al., 2002) and dialyzed against distilled water for 4 days to remove low molecular weight compounds. The crude polysaccharides were obtained through precipitation with ethanol to the final concentration of 90%, and overnight at 4℃. The precipitate was collected as the crude CM-hs-CPS and lyophilized.

Isolation and purification of polysaccharides

The crude polysaccharide, CM-hs-CPS (100 mg), was dissolved in 10 ml distilled water, centrifuged at 8000 rpm for 10 min, and loaded onto a DEAE-cellulose-52 column (2.6 × 30 cm). After loading with sample, the column was eluted with 80 ml in order of distilled water, 0.1 mol/L NaCl, 0.2 mol/L NaCl and 0.3 mol/L NaCl, respectively, at flow velocity of 1.0 ml/min. The elute was collected at 4 ml/tube. This process was monitored by the phenol-sulfuric acid method (Xu et al., 2005). The resulting fraction (5 mg) was loaded onto a Sephadex G-100 column (2.6 × 30 cm) for purification, and then eluted with distilled water. Consequently, the homogeneous fraction CM-hs-CPS2 was obtained and lyophilized.

Polysaccharides ultraviolet spectroscopy

The purified CM-hs-CPS2 was dissolved in distilled water to the concentration of 0.1 mg/ml, and scanned with UV spectrophotometer (UV-2450, SHIMADZU) in wavelengths from 800 to 200 nm.

GC analysis

Derivatives of acid hydrolytic products from CM-hs-CPS2 were analyzed by gas chromatography (GC) to identify the monosaccharide components. The CM-hs-CPS2 fraction (10 mg) was placed in an ampoule, hydrolyzed with 3 ml of 4 mol/l trifluoroacetic acid at 115℃ for 12 h. The ampoule was filled with nitrogen and sealed. The acidolysis solution was dried with a stream of N_2 at 65℃ in a water bath. The solid residual was re-dissolved in methanol, and then distilled at 65℃ with a stream of N_2. This process was repeated three times to remove the acid. The solid hydrolysate was mixed with hydroxylamine hydrochloride and inositol as an internal standard. Then pyridine added to dissolved with shaking at 95℃ for 30 min. After that, the sample was cooled down to room temperature, and then added acetic anhydride to carry on acetylation reaction at 95℃ for 30 min. Following, the solution was mixed with methanol, and dried with a stream of N_2. The derivatives were dissolved in chloroform and analyzed by GC. The derivation process of mixed standard monosaccharide (rhamnose, arabinose, xylose, mannose, glucose, galactose) was operated with the same method mentioned above. The GC was performed on Agilent 6820 GC (Agilent Corporation) equipped with a flame ionization detector (FID), through a fused-silica capillary column (0.23 mm × 30 m). N_2 was used as carrier gas, and H_2 as burning gas. Sample injection volume was 1 µl on condition of N_2 flow of 50 ml/min at split ratio of 50:1. Temperatures of injection and FID detector were controlled at 230℃. The column temperature was first fixed at 130℃ for 20 min, increased to 190℃ at 5℃/min, and maintained for 20 min, then increased to 230℃ with the ratio of 5℃/min for 10 min.

IR analysis

Infrared (IR) spectrometry of CM-hs-CPS2 was performed at 4000 - 400 cm^{-1} wave range. The dried CM-hs-CPS2 was pressed into KBr disks, and then scanned with a Fourier transform infrared (FT-IR) spectrometer (Tensor 27, BRUKER).

In vitro antioxidant assay

DPPH radical scavenging assay

The DPPH radical scavenging capacity was assayed according to the method of Luo et al. (2009) with slightly modification. Each sample at different concentrations (2, 4, 6 and 8 mg/ml) was mixed with 2 ml of 0.04 mg/ml DPPH· in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, it was centrifuged at 5000 rpm for 10 min. After that, the absorbance of the supernatant was measured at 517 nm. Vitamin C (Vc) was used as the positive control. The DPPH radical scavenging capacity was calculated by the following formula:

$$\text{Scavenging capacity (\%)} = \frac{1-(A_1-A_2)}{A_0}\times 100$$

Where A_0 is the absorbance of the control (ethanol sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the blank.

Reducing power assay

The reducing power was determined by the method of Tsais (2006). Briefly, 1 ml sample of different concentration (2, 4, 6 and 8 mg/ml) in phosphate buffer (0.2 mol/l, pH 6.6) was mixed with 2 ml potassium ferricyanide (1%, w/v), and incubated at 50℃ for 20 min. After that, 2 ml of trichloroacetic acid (10%, w/v) was added to mixture and stop the reaction. After centrifugation at 5000 rpm for 10 min, the supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml ferric chloride (0.1%, w/v). Ten minutes later, the absorbance was measured at 700 nm. Vitamin C (Vc) was used as the positive control.

Ferrous ion chelating capacity assay

The ferrous ion chelating capacity was determined follow by the

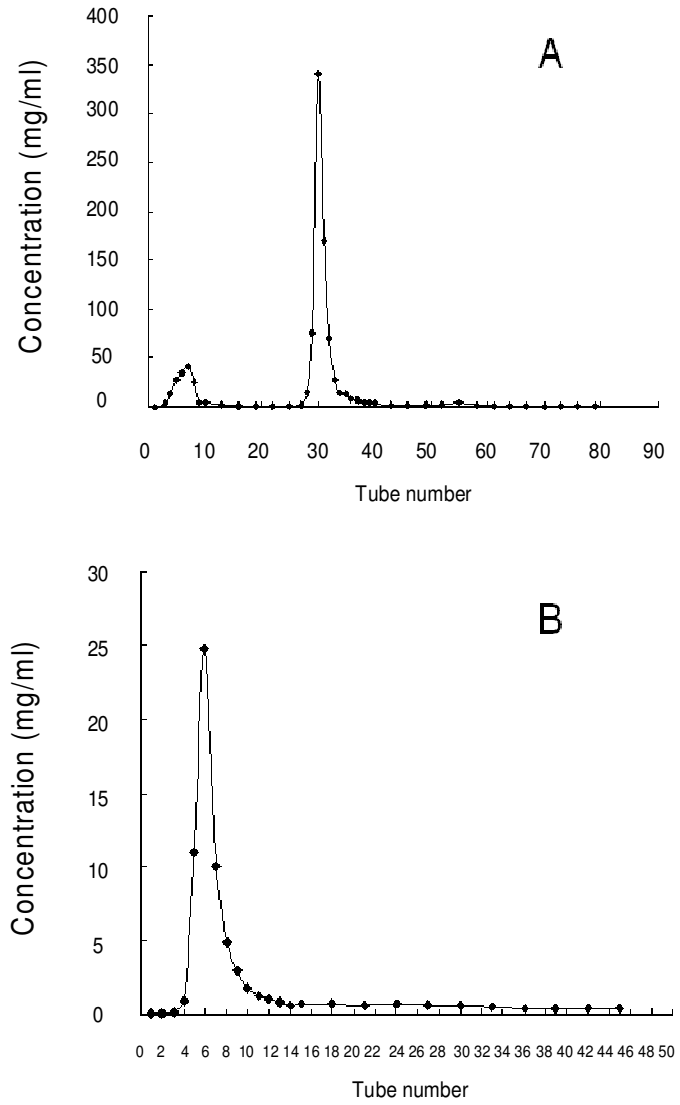

Figure 1. Elution profiles of polysaccharides extracted from cultured C. militaris by column chromatography. (A) Ion exchange chromatogram of the crude polysaccharides, CM-hs-CPS, on a DEAE-cellulose-52 column. (B) Gel filtration chromatogram of the acidic polysaccharide fraction, CM-hs-CPS2, on a Sephadex G-100 column.

method of Decker (1990) with slightly modification. The reaction mixture, containing 3 ml of sample with different concentrations (2, 4, 6 and 8 mg/ml), 0.05 ml of 2 mmol/L ferrous chloride ($FeCl_2$) solution and 0.2 ml of 5 mmol/L ferrozine solution, was shaken vigorously and incubated at room temperature for 10 min. The absorbance of the mixture was then measured at 562 nm. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as the positive control. The ferrous ion chelating capacity of sample was calculated as follows:

$$\text{Chelating capacity (\%)} = \frac{A_0-(A_1-A_2)}{A_0}\times 100$$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the blank.

Statistical analysis

All treatments and assays were carried out in triplicates for three separate experiments. Values are represented as mean±SD (standard deviation).

RESULTS

Isolation and purification of crude CM-hs-CPS

The crude polysaccharide, CM-hs-CPS, was obtained from fruiting bodies of cultured *C. militaris* by hot water extraction and ethanol precipitation. Figure 1 shows the elution profile of the deproteinized CM-hs-CPS on the DEAE-cellulose-52 ion exchange column. The first peak eluted with the distilled water is ascribed to a neutral polysaccharide fraction. The main peak eluted with NaCl solution is an acidic polysaccharide fraction, termed CM-hs-CPS2. This fraction was collected and applied to the Sephadex G-100 gel filtration column, resulting in the elution profile in Figure 1. CM-hs-CPS2 was eluted as a single peak. The UV absorption spectra (Figure 2) of CM-hs-CPS2 showed no absorption at 260 and 280 nm, indicated that nucleic acid and protein were absent in this polysaccharide.

Structural composition of CM-hs-CPS2

GC analysis of CM-hs-CPS2

The alditol acetates derived from the acetylation of CM-hs-CPS2 hydrolysate and standard monosaccharide were measured by gas chromatography (GC). As shown in Figure 3, mixture of monosaccharide and inositol were separated completely. The peaks emerged in the order of rhamnose, arabinose, xylose, mannose, glucose, galactose and inositol. Three monosaccharides, termed mannose, glucose and galactose were identified in the hydrolysate of CM-hs-CPS2 on the basis of the standard monosaccharide retention time and correction factor (Figure 3). According to the peak area, the mole ratio of mannose:glucose:galactose in CM-hs-CPS2 was 1.35: 8.34:1.00.

IR analysis of CM-hs-CPS2

Infrared (IR) spectrometry of CM-hs-CPS2 showed that the largest absorption peak at 3396 cm^{-1} is ascribed to the stretching of the hydroxyl (OH) group and the hydrogen bond within or between the molecules (Figure 4). The peak at 2929 cm^{-1} is ascribed to the C-H stretching band of the saccharide. And the weak peaks

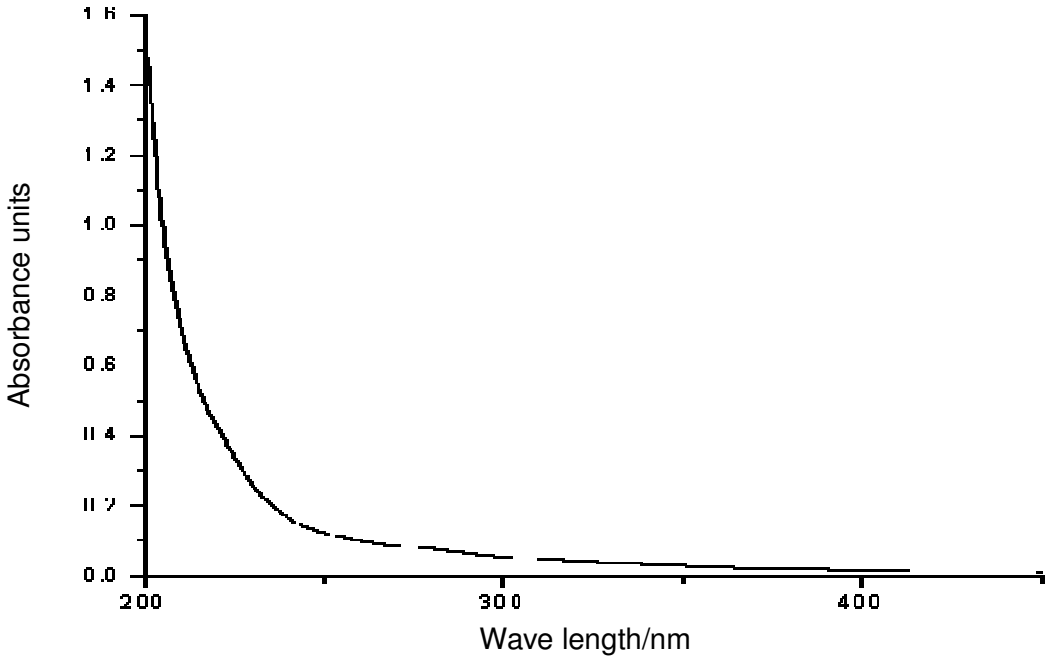

Figure 2. Ultraviolet absorption curve of CM-hs-CPS2.

between 1400 and 1200 cm^{-1} are ascribed to C-H bending vibration. The bands at 1651 and 1541 cm^{-1} are assigned to stretching vibration of the C=O bond and the bending vibration of the N-H bond, which suggests the presence of acetamido group. The two absorption bands near 1240 cm^{-1} and 850 cm^{-1} resemble the stretching band of S=O and C-O-S, respectively, which indicates the existence of $–O-SO_3$. There is a group of strong absorption peaks from 1200 to 950 cm^{-1}, which can be attributed to the ether linkage (C-O-C) and the hydroxyl presented in the ring of pyranose. And the peak at 761 cm^{-1} was the symmetric ring stretching of pyranose, impling the monosaccharide composing CM-hs-CPS2 was pyranose. The presence of the α-glycosidic linkage was proven by the C-H bending vibration at 848 cm^{-1}. And the absorption peaks at 931 cm^{-1} and 761 cm^{-1} imply the existence of α-D-glucopyranose (α-D-Glcp).

In vitro antioxidant activities of CM-hs-CPS2

DPPH radical scavenging activity of CM-hs-CPS2

As depicted in Figure 5, CM-hs-CPS2 exerted concentration-dependent DPPH· scavenging activity. At a concentration of 8 mg/ml, the DPPH· scavenging activity of CM-hs-CPS2 reached to 89%. Compared with positive control (Vc), the scavenging activity of CM-hs-CPS2 was a little lower than Vc at each concentration. These results indicated that CM-hs-CPS2 had strong DPPH· scavenging activities.

Reducing power of CM-hs-CPS2

The reducing power is an important measure of the antioxidant capability of antioxidant. It was determined by absorption spectrometry to monitor the transformation of Fe^{3+} to Fe^{2+} in the presence of the antioxidant. The increasing absorbance is correlated with the increasing reducing power. As shown in Figure 6, the sample showed a dose-dependent reducing capacity. The reducing power of CM-hs-CPS2 was 1.188 at the concentration of 8 mg/ml, compared with the Vc at 2.108. It was obvious that the reducing activity of CM-hs-CPS2 was much lower than that of Vc, suggesting CM-hs-CPS2 had a moderate reducing power.

Ferrous ion chelating activity of CM-hs-CPS2

The ferrous ion chelating capacity is also one of the important antioxidant properties. Transition metals such as Fe^{2+} are known to play key roles in lipid peroxidation by generating hydroxyl radicals through Fenton reaction. In the assay, the ferrous ion chelating capacity of the antioxidants was detected by inhibiting the formation of red-colored ferrozine- Fe^{2+} complex.

The results in Figure 7 showed that the ferrous ion chelating activity of CM-hs-CPS2 was 85% at a concentration of 8 mg/ml. It is notable that the positive control of EDTA-2Na has excellent ferrous ion chelating capacities with 100% at each concentration. As a result, CM-hs-CPS2 exhibited strong Fe^{2+} chelating activities at

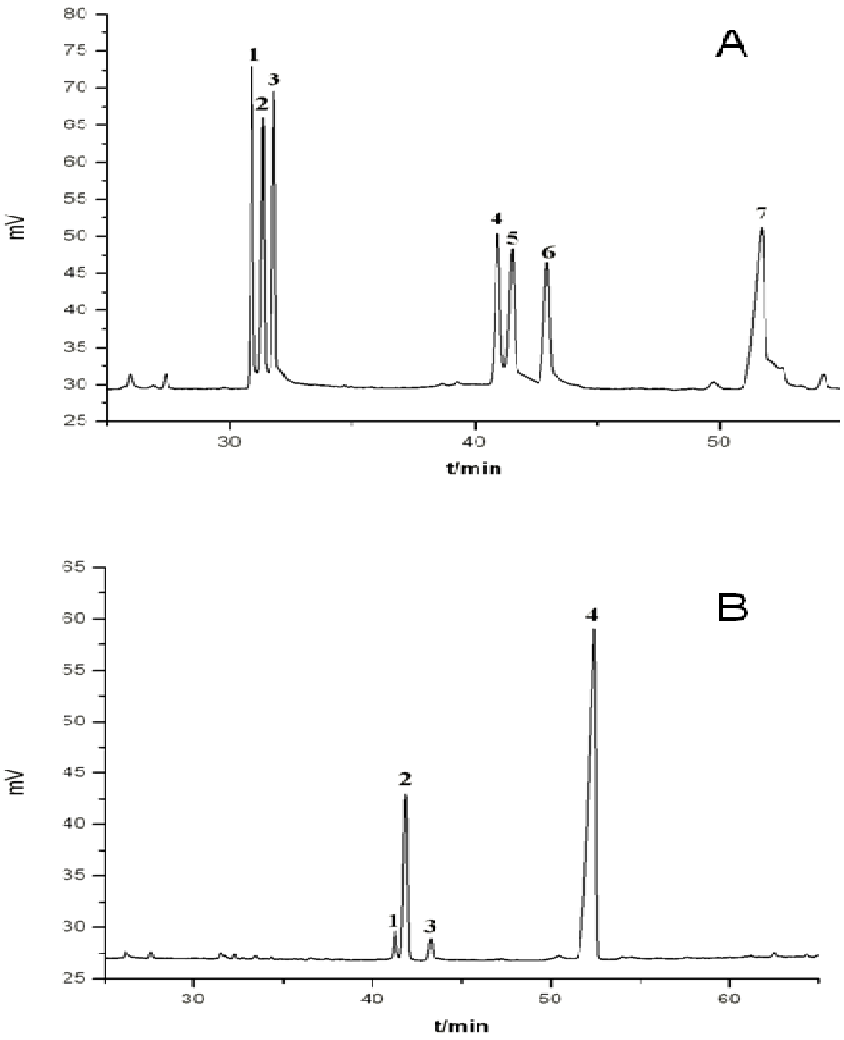

Figure 3. GC profile of standard monosaccharide (A) and CM-hs-CPS2 (B). (A) Peak identity: 1, Rhamnose (rt: 30.897); 2, Arabinose (rt: 31.336); 3, Xylose (rt: 31.803); 4, Mannose (rt: 40.963); 5, Glucose (rt: 41.530); 6, Galactose (rt: 42.978); 7, Inositol as an internal standard. (B) Peak identity: 1, Mannose (rt: 41.249); 2, Glucose (rt: 41.862); 3, Galactose (rt: 43.272); 4, Inositol as an internal standard.

the concentration of 4, 6 and 8 mg/ml.

DISCUSSION

Currently, cultivated fruiting bodies of *C. militaris* are commercially available as medicine materials and health food products in China, Korea, and South East Asia (Li et al., 2006). Biologically active polysaccharides are widespread among mushrooms, and most have unique structures in different species. As a result of this phenomenon, several studies have been conducted to determine accurately the structures of these different polysaccharides.

In this study, CM-hs-CPS2 was extracted from fruiting bodies of *C. militaris* which were cultivated on solid rice medium. The results of its structure composition exhibited that CM-hs-CPS2 was composed by mannose, glucose and galactose in the mole ratio of 1.35:8.34:1.00. The IR spectrum results revealed that the CM-hs-CPS2, composed by pyranose and linked by α-glycosidic linkage, was a kind of sulfating acidic polysaccharide containing acetamido-group. In the last few years, the structural characterizations of several polysaccarides obtained from cultured *C. militaris* were reported. Two polysaccharides (CPS-2 and CPS-3) were isolated from cultured *C. militaris* by Yu and his group. CPS-2 was made mainly of rhamnose, glucose and galactose in a mole ratio of 1:4.46:2.43. CPS-3 was a homogeneous polysaccharide, which contained glucose with a major linkage form of α-D-glucose (Yu et al., 2004). After that, polysaccharide P70-1 was also obtained from the fruiting

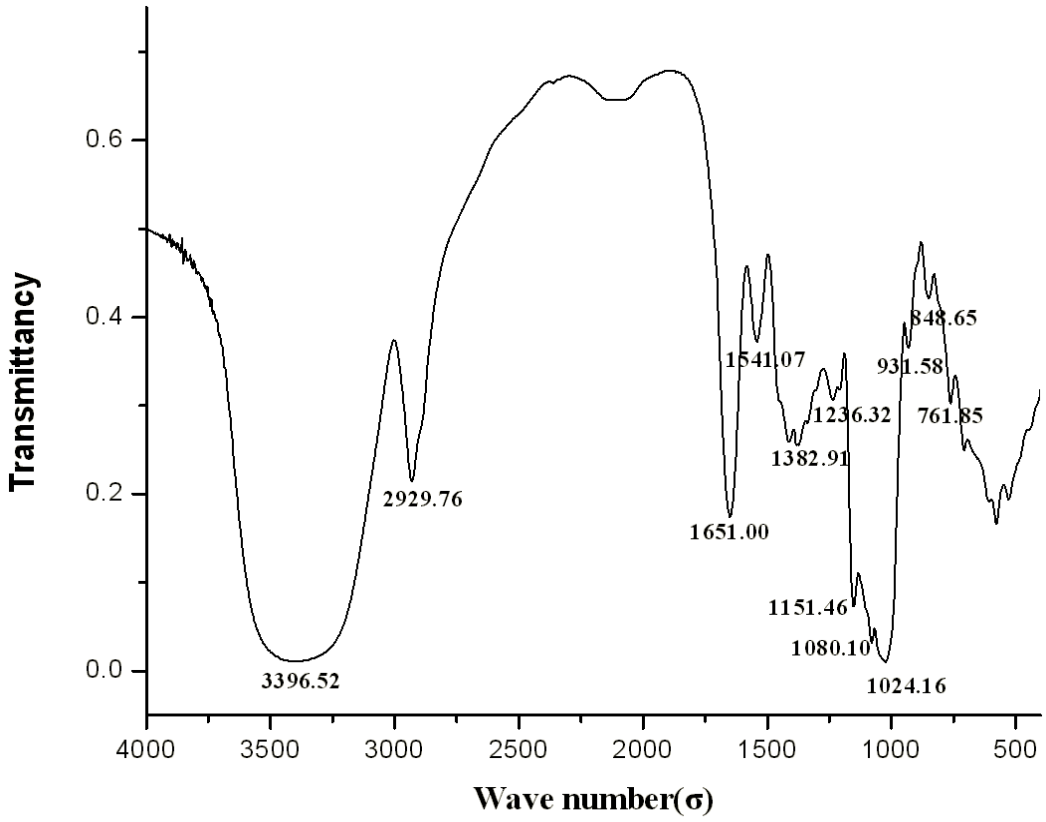

Figure 4. IR spectrum of CM-hs-CPS2.

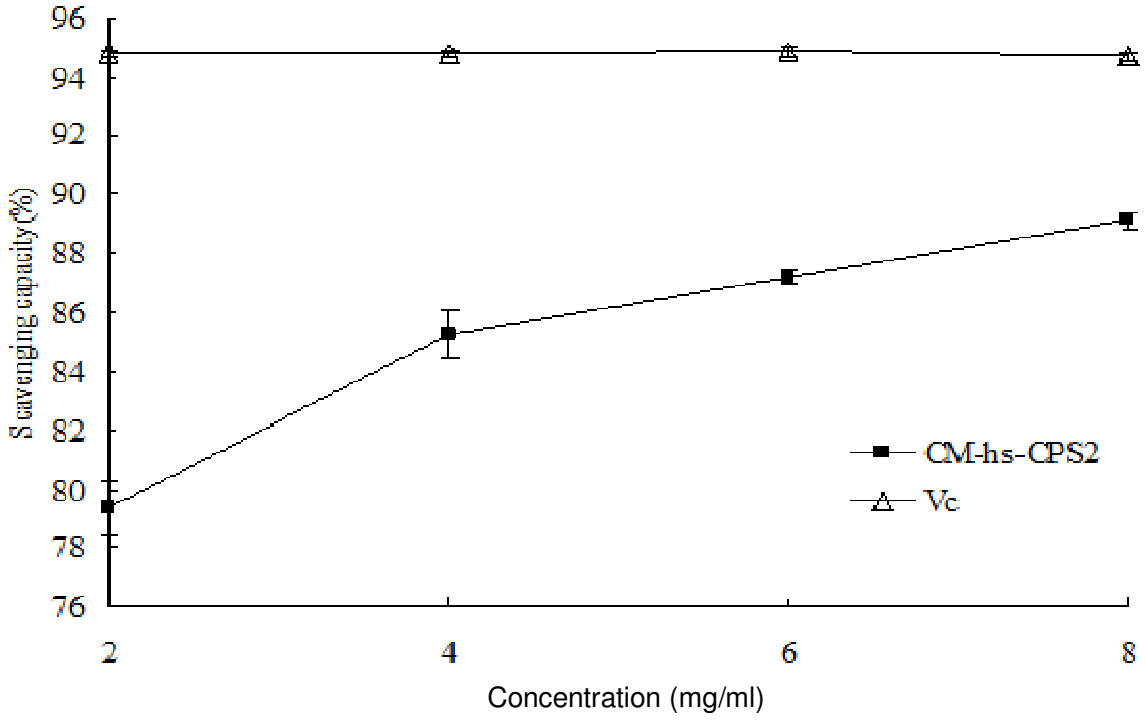

Figure 5. The DPPH radical scavenging activities of CM-hs-CPS2, with Vc as the positive control. Values are represented as mean±SD (n=3).

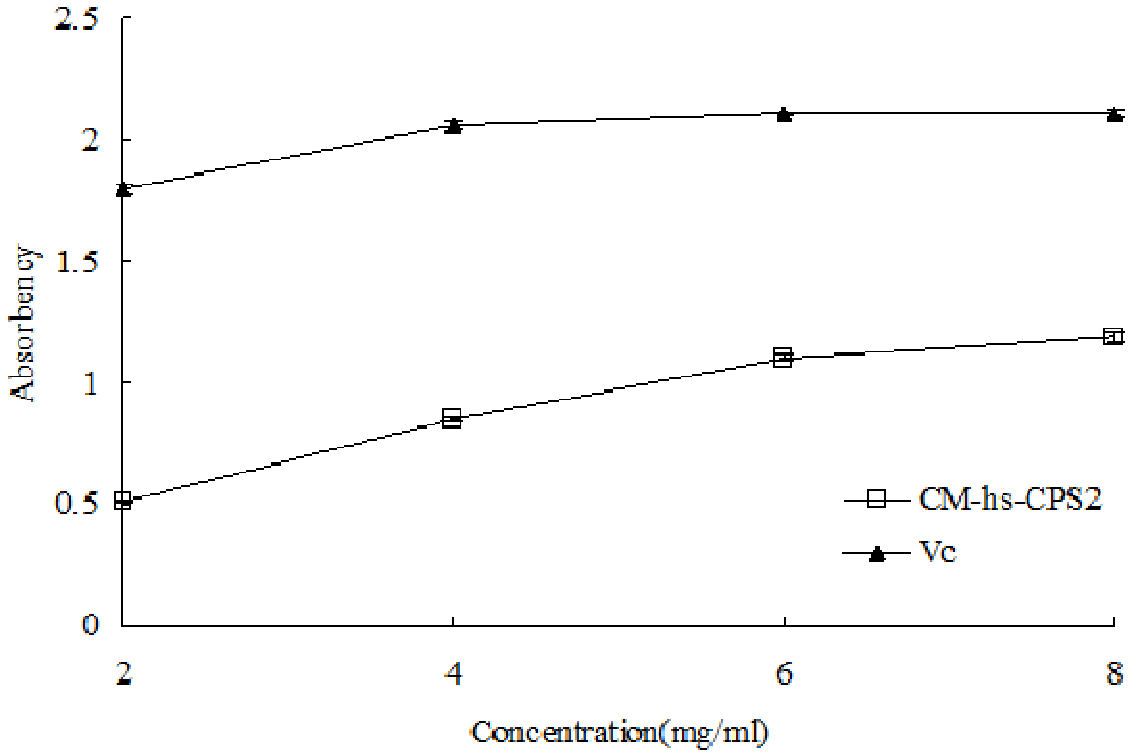

Figure 6. The reducing power assay of CM-hs-CPS2, with Vc as the positive control. Values are represented as mean±SD (n=3).

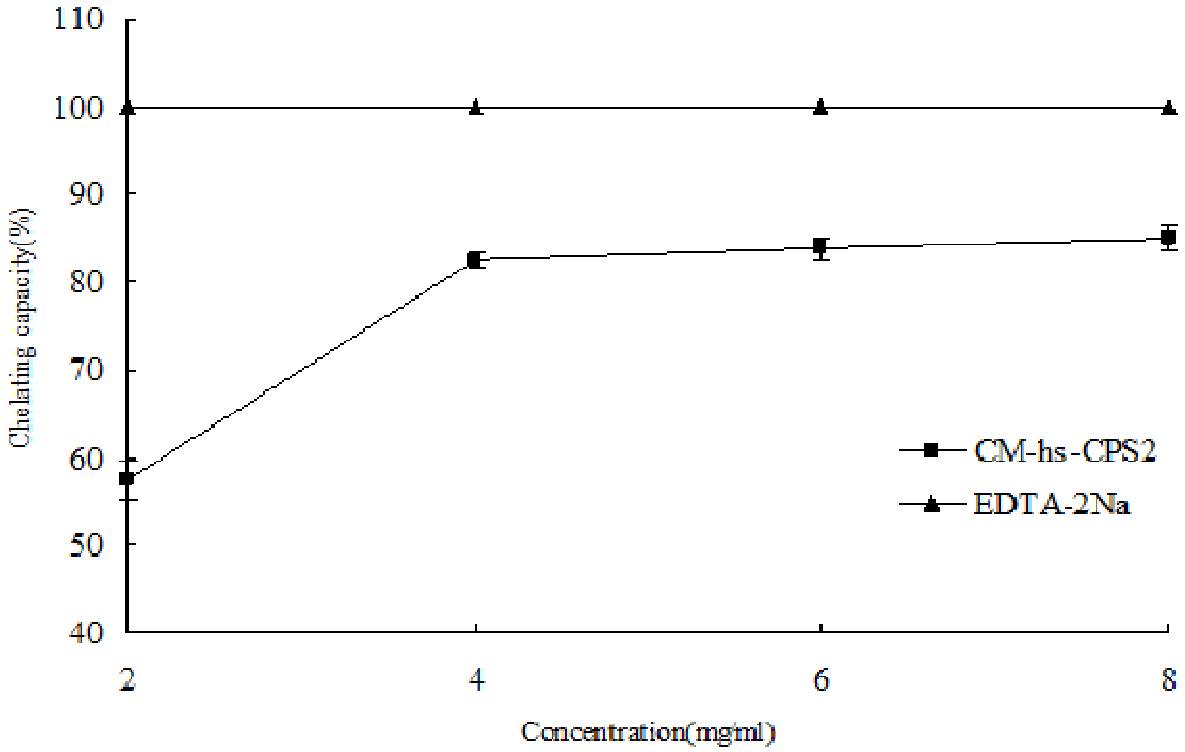

Figure 7. The ferrous ion chelating capacity of CM-hs-CPS2, with EDTA-2Na as the positive control. Values are represented as mean±SD (n=3).

bodies of cultured *C. militaris* (Yu et al., 2007). An acidic polysaccharide isolated from *C. militaris* grown on germinated soybeans was composed of galactose, arabinose, xylose, rhamnose and galacturonic acid (Ohta et al., 2007). Lee and his colleagues have reported the structural properties of CPMN Fr II and CPMN Fr III which were obtained from cultured mycelia of *C. militaris*. CPMN Fr II was a 1, 6-branched-glucogalactomannan with the β-linkage and random coil conformation (Lee et al., 2010a). CPMN Fr III was a β-1, 4-branched-β-1, 6-galactoglucomannan (Lee et al., 2010b). The interpretation of polysaccharide structural differences from above results may relative to the fact that they were different *C. militaris* strains. Furthermore, different culture medium (silkworm pupa, solid rice medium and broth) may contribute to different structure of the polysaccharide.

The antioxidant properties of natural products are most relevant to their health protecting and anti-cancer functions. Firstly, CM-hs-CPS2 was able to scavenge the stable free radical DPPH efficiently. This may attributed to their electron transfer or hydrogen donating ability. It has been suggested the existence of hydroxyl group in polysaccharides could donate electrons to reduce the radicals to a more stable form or reacts with the free radicals to terminate the radical chain reaction (Leung et al., 2009). There was a direct correlation between antioxidant activities and reducing power (Duh et al., 1999). Secondly, the presence of reductant associated with the reducing power. Reductant has been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Zhang et al., 2010). As a result, CM-hs-CPS2 showed a moderate reducing power, which may due to the presence of -OH in its structure. Thirdly, the results of the ferrous ion chelating capacity assay showed CM-hs-CPS2 exhibited strong Fe^{2+} chelating activities. It has been demonstrated that compounds with metal chelating activities usually contain two or more of the following functional groups: -OH, -SH, -COOH, $-PO_3H_2$, -C=O, $-NR_2$, -S- and -O- (Yuan et al. 2005). Accordingly, the ferrous ion chelating capacities of CM-hs-CPS2 was partially accounted for the presences of –OH, C=O, -S- and -O- groups in its structure. Moreover, it has reported that the antioxidant capacity of polysaccharides also depends strongly on the type and organization of the monosaccharide, the linkage pattern of the main chain (alpha or beta) and the branching configuration (Liu et al., 2007).

The fundamental findings in this study are beneficial to the interpretation in the connection of the polysaccharide structures and its biological activities. Due to the complex structures of bioactive polysaccharides, it is difficult to elucidate their chemical properties and relationships between their structure and activity. Therefore, great efforts should be devoted to reveal the structure-activity relationship of polysaccharides from *C. militaris* in further study.

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