Purification of polysaccharides from *Cordyceps militaris* and their anti-hypoxic effect

YUAN DONG[1,2], SHUANG HU[1], CHUNGANG LIU[1], QINGFAN MENG[1], JIA SONG[1], JIAHUI LU[1], YINGKUN CHENG[1], CHAOHUI GAO[1], YAN LIU[1], DI WANG[1] and LIRONG TENG[1,3]

[1]College of Life Science, Jilin University, Changchun, Jilin 130012; [2]Academy of Laboratory, Jilin Medical College, Jilin, Jilin 132013; [3]College of Life Science, Zhuhai College, Jilin University, Zhuhai, Guangdong 519000, P.R. China

Received January 20, 2014; Accepted September 4, 2014

DOI: 10.3892/mmr.2014.2786

Abstract. Acute mountain sickness, one of the most common altitude diseases, causes lung and brain injury. The present study aimed to investigate the anti-hypoxic effect of purified polysaccharides extracted from *Cordyceps militaris*. The aqueous extract of *Cordyceps militaris* was purified progressively through a DEAE-52 cellulose anion exchange column and a Sepharose G-100 column. The fraction CMN1, with a molecular weight of 37842 Da, was the main fraction obtained and its chemical composition and structural characteristics were determined. CMN1 was found to have a monosaccharide composition of L-rhamnose, L-arabinose, D-mannose, D-galactose. The backbone of CMN1 comprised (1→2) and (1→3) linkages, with branched (1→6) and (1→4) linkages. The anti-hypoxic effects of CMN1 were determined using a sodium nitrite toxicosis test, acute cerebral ischemic/hypoxic test and normobarie hypoxia test. CMN1 (0.5 g/kg) possessed a similar anti-hypoxic effect to rhodiola oral liquid. Overall, the *Cordyceps militaris* polysaccharide, CMN1, was identified as an effective agent against hypoxia.

Introduction

As one of the most common altitude illnesses, acute mountain sickness (AMS) causes lung and brain injury, with symptoms including headache, loss of appetite, dizziness and insomnia (1). The decreased barometric pressure and subsequent reduction in available oxygen are the primary causal factors of AMS (2). Long-term hypoxia causes irreversible damage and ultimately leads to organ failure. Currently, the normal vasodilating agents, nifedipine and acetazolamide, are used as prophylactic agents to reduce the incidence and severity of AMS at high altitudes (3). However, serious adverse effects, including headache and cardiopalmus have been observed during the clinical treatment (4), creating a demand for the identification of alternative agents.

Cordyceps militaris, which belongs to the class *Ascomycetes* and the Dong Chong Xia Cao group of Chinese herbs, possesses potential antioxidant (5), immunomodulatory (6), antitumor and anti-inflammatory properties (7). In Western countries, *Cordyceps militaris* is considered to be a Chinese herb with anti-aging and anticancer effects (8). Several studies have investigated the polysaccharides of *Cordyceps,* which are a rich and important activity group. One *Cordyceps militaris* polysaccharide-enriched fraction produced hypoglycemic activity (9) and reduced plasma glucose in normal Wistar-Kyoto rats (10). In our previous study, *Cordyceps militaris* polysaccharides exhibited significant anti-diabetic and anti-nephropathic activities (11). As reported previously, the bioactivities of polysaccharides are associated with their chemical composition, glycosidic linkages, conformation, molecular weight and degree of branching (12). Previous studies have examined different polysaccharides extracted from the *Cordyceps militaris* sporocarp and these are comprised mainly of mannose, rhamnose, galactose and glucose and have an average molecular weight of 30 kDa (13-15). However, few studies have investigated the purification and bioactivities of polysaccharides separated from *Cordyceps militaris* mycelium, which is cultured by submerged fermentation (16,17). Submerged fermentation presents the advantage of simultaneously enabling a decrease in disposal costs and the production of value-added products and, thus, has been widely used.

In the present study, polysaccharides extracted from *Cordyceps militaris* mycelium were purified and characterized. Furthermore, the anti-hypoxic effects of the purified *Cordyceps* polysaccharides were detected *in vivo*.

Materials and methods

Strain and reagents. The study was approved by the ethics committee of the Lab Animal Center of Jilin University; ref no. 2009-0011). *Cordyceps militaris* was purchased from the National Biological Resource Center (Chiba, Japan; cat

Correspondence to: Dr Di Wang, College of Life Science, Jilin University, 2699 Qianjin Street, Changchun, Jilin 130012, P.R. China
E-mail: jluwangdi@gmail.com

Professor Lirong Teng, College of Life Science, Zhuhai College, Jilin University, 286 Jinhai Road, Zhuhai, Guangdong 519000, P.R. China
E-mail: tenglr@jlu.edu.cn

Key words: Cordyceps militaris, polysaccharides, purification, structural analysis, anti-hypoxic effect

no. NBRC9787). Sodium nitrite, glucose, peptone, yeast extract powder, H_2SO_4, $NaIO_4$, acetonitrile, glycol (analytical grade), NaOH, potassium hydrogen phthalate, acetic acid, potassium borohydride, $BaCO_3$, KH_2PO_4, $MgSO_4.7H_2O$, $(NH4)_2SO_4$, $ZnCl_2$, Vitamin B1 and dextran standards were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

Submerged incubation and fermentation. *Cordyceps militaris* was cultured in a rotary shaker incubator (10 L, Biostat B; Sartorius AG, Goettingen, Germany) at 150 rpm for 5 days at 26˚C. The cultured medium contained 20 g/l glucose, 10 g/l peptone, 18 g/l yeast extract powder, 3 g/l KH_2PO_4, 3 g/l $MgSO_4$ $7H_2O$, 10 g/l $(NH4)_2SO_4$, 0.01 g/l $ZnCl_2$ and 0.24 g/l vitamin B1. The mycelia were then harvested and lyophilized for further use.

Crude extract preparation and preliminary identification. As described previously (18), the aqueous extract from *Cordyceps militaris* was prepared by extracting 100 g mycelia powder twice using hot water at 80˚C for 3 h. Following centrifugation at 4,500 x g for 10 min, the supernatant was sequentially concentrated and freeze-dried to produce the solid aqueous extract of *Cordyceps militaris* (CM (indicating the *Cordyceps militaris* extract)). According to the previously described method (19), different chromogenic reactions were used to determine the constituents of CM.

Purification of the CM. Using Sevag reagent [V (n-butanol): V (chloroform)=1:4, 50 ml, Sigma-Aldrich, St. Louis, MO, USA], the proteins present in the CM were removed (20). Ethanol (4-fold) was added to the supernatant, which was placed at 4˚C overnight. The precipitation was then dissolved in double distilled (DD) water and placed in a 2.6 cm x 35 cm DEAE-52 cellulose anion exchange column (21). The column was eluted with DD water, followed by 0.1 and 0.3 mol/l NaCl, respectively, at a flow rate of 1 ml/min. The polysaccharide fraction was collected and detected using an anthrone acid method (19). The gel permeation chromatography system, Sepharose G-100 (General Electric Co.Salt Lake City, Utah, USA) was used for further purification. The column was eluted with DD water at a flow rate of 0.4 ml/min. The fractions (10 ml each) were then collected (13) and freeze-dried.

Fourier transform infrared spectroscopy (FTIR) determination. The purified polysaccharides (4 mg) were ground thoroughly using 150 mg KBr (Sigma-Aldrich). The average transmission spectra (n=50) were recorded using an IRPrestige-21 FTIR spectrometer (Shimadzu Corporation, Tokyo, Japan) between 400 and 4,000 cm^{-1} and the absorbance was determined.

Homogeneity and molecular weight determination. The homogeneity and molecular weight were analyzed using a high performance liquid chromatography (HPLC)/evaporative light scattering detector (ELSD) system (22). An LC-10ATvp HPLC system (Shimadzu Corporation) equipped with a TSKgel G4000PWXL column (Tosoh, Tokyo, Japan) and an Alltech 2000ES ELSD (Shimadzu Corporation) was used. Briefly, DD water served as the mobile phase, which was driven by a double pump (Waters 150; Millipore, Billerica, MA, USA) at a flow rate of 0.45 ml/min. The aerosol level was 60%, the drift tube temperature was 120˚C and the nebulizing nitrogen pressure was 25 psi. Dextran standards were used to create a calibration curve, as previously described (23).

Monosaccharides analysis. The analysis of monosaccharides was investigated according to the previous method (24). The polysaccharide (20 mg) was hydrolyzed with 1 M H_2SO_4 (1 ml) for 6 h at 105˚C in a sealed glass tube and the pH was adjusted to 7.0 using $BaCO_3$. The solution was then centrifuged at 3,200 x g for 10 min to separate the hydrolysates, which were further analyzed using the HPLC/ELSD system. The chromatograph was fitted with a Prevail™ ES carbohydrate analysis column (Alltech Associates, Inc., Deerfield, IL, USA), which was eluted with 75% acetonitrile (Sigma-Aldrich) at a flow rate of 1.0 ml/min. The results were compared with the following monosaccharide standards: D-glucose, L-rhamnose (Rha), D-xylose, D-galactose (Gal), D-mannose (Man) and L-arabinose (Ara), trehalose (Sigma-Aldrich) (25).

Periodate oxidation-Smith degradation reaction of polysaccharides. The polysaccharide (20 mg) was dissolved in 15 mM $NaIO_4$ (25 ml; pH 4) in darkness at 4˚C. Subsequently, 100 μl was withdrawn at 6 h intervals, diluted with distilled water and measured spectrophotometrically at 223 nm until a stable absorbance was reached (26). The consumption of HIO_4 was calculated and the production of formic acid was determined by titration with 0.005 M NaOH and glycol (2 ml) was added to terminate the oxidation reaction. The remaining periodate product was then fully dialyzed against DD water for 48 h. This dialysate was concentrated and reduced with potassium borohydride (70 mg) overnight at room temperature and the pH was adjusted using acetic acid (Sigma-Aldrich) to pH 7.0. The solution was dialyzed against DD water for 24 h and 3 ml sample was further analyzed using the HPLC/ELSD system. The remaining product was hydrolyzed using 1 M H_2SO_4 at 25˚C for 40 h and was adjusted to pH 7.0 using $BaCO_3$. Following centrifugation at 3,200 x g for 10 min, the hydrolysates were analyzed using HPLC/ELSD under the same conditions that were used for the monosaccharide composition analysis.

In vivo experiments in an animal model of hypoxia. The experimental animal procedure was approved by the Lab Animal Centre of Jilin University [Changchun, China; License no. SCXK-(JI) 2006-0001)]. Mice weighing 18-22 g were maintained under a constant 12 h light/dark cycle at 23±1˚C, with a humidity of 60±2% with water and food available *ad libitum*. All mice were fed with standard laboratory feed in an animal room for 3 days prior to the experiments. To produce experimental models of hypoxia, 180 mice (90 males and 90 females) were randomly divided into three groups for the normobarie hypoxia test, sodium nitrite toxicosis test and acute cerebral ischemic/hypoxic test (27), respectively. The normobarie hypoxia test was performed in a sealed wide-mouth bottle containing soda lime (Sigma-Aldrich). The sodium nitrite toxicosis test was performed by injection of 2 g/ml sodium nitrite. The acute cerebral ischemic-hypoxic test was performed by decapitation of the mice. In all three tests, mice received either physiological saline or 1.5 ml/kg/day rhodiola oral liquid, which served as the normal control (NC) group or the positive

control (PC) group. The remaining mice were divided into three groups (n=12) and treated orally with CMN1 (0.0 g/kg, 0.2 g/kg or 0.5 g/kg) once a day. After 24 days, the survival rate of hypoxia under normal pressure and following sodium nitrite injection were determined and the persistence of gasping following decapitation was examined.

Statistical analysis. All data are expressed as the mean ± standard deviation. Data were evaluated using one-way analysis of variance to detect statistical significance, followed by post-hoc multiple comparisons (Dunn's test). P≤0.05 was considered to indicate a statistically significant difference.

Results

Phytochemical assessment. The constituents of the *Cordyceps militaris* extract, including proteins, organic acid and sugars were detected (Table I). At room temperature, organic acid was unstable, which suggested that the major effective components in the *Cordyceps militaris* extract were protein and polysaccharides. In addition, the content of the total polysaccharides was 163 mg/g in the cultured *Cordyceps militaris* mycelium.

Purification and characterization of polysaccharides. The neutral or acidic polysaccharides in *Cordyceps militaris* water extracts were separated using anion exchange chromatography in a DEAE-cellulose column. Two fractions, CM1 and CM2, were eluted using DD water and 0.1 M NaCl, respectively and the yields of CM1 and CM2 were 0.475 and 0.025 g/g, respectively (Fig. 1A). When examining the rate of production, only CM1 was further purified using the gel permeation chromatography system Sepharose G-100. A single elution peak (CMN1) appeared at 60 min (Fig. 1B) with a molecular mass of 37,842 Da (Fig. 2A and B). Rha, Ara, Man and Gal were present in CMN1, according to the retention time in the HPLC figure print (Fig. 2C and D). The molar ratio of Rha, Ara, Man and Gal was 1.48:11.34:11.62:1.00.

Furthermore, the characteristic structure of CMN1 was analyzed using FTIR and periodate oxidation-Smith degradation. In the FTIR spectra (Fig. 3), peaks at 3,494, 2,900, 1,627, 1,558, 1,458, 1,006 and 524 cm^{-1} were noted.

Periodate oxidation-Smith degradation was performed to confirm the linkage mode of the glucose present in CMN1 (Fig. 4) and the HPLC method was performed to analyze the products following Smith degradation. Rha and Ara were noted prior to hydrolysis, however, Ara, glycerol and erythritol were observed following hydrolysis (Fig. 4B).

Anti-hypoxic effects of CMN1. In the normobarie hypoxia test and acute cerebral ischemic-hypoxic test, mice were sacrificed by suffocation. In sodium nitrite-induced acute acquired methemoglobinemia, less oxygen is carried by hemoglobin, which leads to tissue death (25). Compared with the NC group, 0.5 g/kg CMN1 improved survival rate by almost 53% (P<0.05) in the sodium nitrite intoxication survival test and by 39% in the hypoxia-resistance test (P<0.05), respectively (Fig. 5A and C). Furthermore, treatment with 0.5 g/kg CMN1 resulted in a 1.88-fold increase in gasping persistence (P<0.01; Fig. 5B). No significant differences were observed between the CMN1-treated and PC groups indicating that CMN1 possesses an anti-hypoxic effect similar to that of rhodiola oral liquid.

Table I. Summary of the preliminary chemical analysis of crude extracts.

Test	CM
Amino acid/polypeptide/protein	+
Soluble reducing sugar	+
Sugars	+
Phenolics/tannin	–
Alkaloids	–
Sterols	–
Terpenes	–
Organic acid	+
Essential oil/oil	–
Anthraquinone	–
Flavonoids	–
Coumarin/lactone	–

'+' detected; '-' undetected; CM, *Cordyceps militaris* extract.

Discussion

AMS, which produces a large number of oxygen radicals, leads to irreversible tissue damage (29). In previous clinical trials, *Sipunculus nudus L*. (27), *Brassica rapa L*. (28), *Rhodobryum giganteum Par*. (30) and rhodiola were used to alleviate the symptoms of AMS. In the present study, *Cordyceps* polysaccharides were purified and characterized and their anti-hypoxic effects were detected *in vivo*.

In the FTIR spectra, a broad stretching peak was observed at ~3,494 cm^{-1} and a weak peak at ~2,900 cm^{-1}. These are characteristic absorption peaks of saccharides and were produced by marked hydroxyl group vibration (31) and by C-H bending vibration of the $-CH_2$ groups (32), respectively. The bands at 1,627 and 1,558 cm^{-1} suggested the existence of a C=O bond and an N-H bond, possibly from the amidogen in Gal. The weak peak at 1,458 cm^{-1} was attributed to vibrations of CH_3 and an absorption peak at 1,006 and 524 cm^{-1}, may have been contributed to by the ether linkage (C-O-C) and the hydroxyl in the pyranose ring.

Analysis of the results obtained in the periodate oxidation-Smith degradation suggested the possible linkage of monosaccharides within CMN1. The consumption of <1 mol periodate (0.82 mol) in the present study indicated the existence of a 1→3 linkage, which does not consume periodate during oxidation. Following periodate oxidation, the presence of Rha and Ala revealed that CMN1 contained a section of (1→3)-linked-Rha and (1→3)-linked-Ala. The monosaccharide ratio suggested that the majority of (1→3)-linked-Ala is located in the backbone and the (1→3)-linked-Rha may be located in the backbone or the side chains. Formic acid production during periodate oxidation confirmed the existence of a 1→6 linkage. The low formic

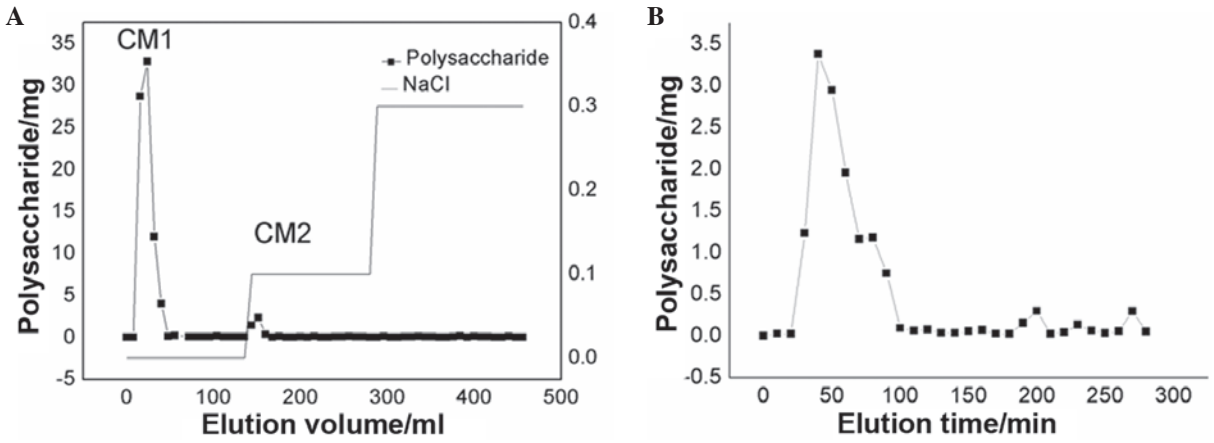
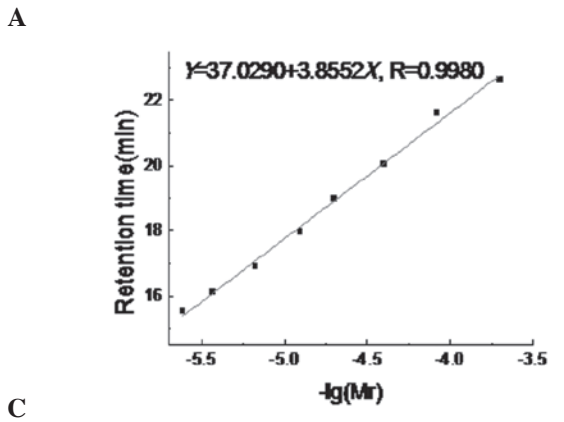
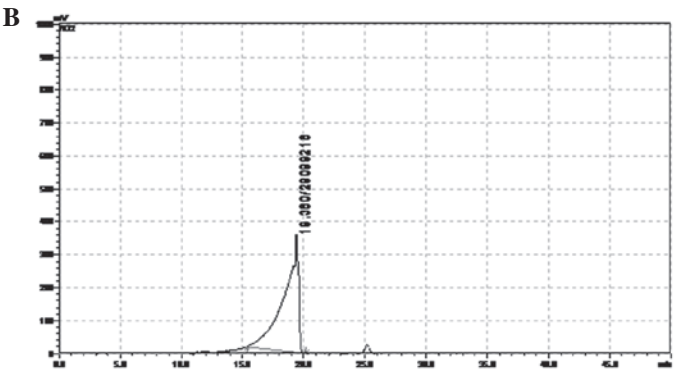


Figure 1. (A) DEAE-Sepharose fast flow chromatogram of the crude polysaccharides. CM1 was eluted using DD water and CM2 was eluted using 0.1 M NaCl at a flow rate of 1.0 ml/min. CM1 (5.0 ml) and CM2 (5.0 ml) were obtained using a fraction collector. (B) CM1 was purified using Sepharose G-100. The column was eluted using DD water at a flow rate of 0.4 ml/min. DD, double distilled; CM, *Cordyceps militaris* extract.





C

Monosaccharide	Retention time (min)
L-rhamnose	6.932
L-arabinose	7.523
D-xylose	8.281
D-mannose	8.928
D-galactose	12.444
D-glucose,	13.445
trehalose	28.735

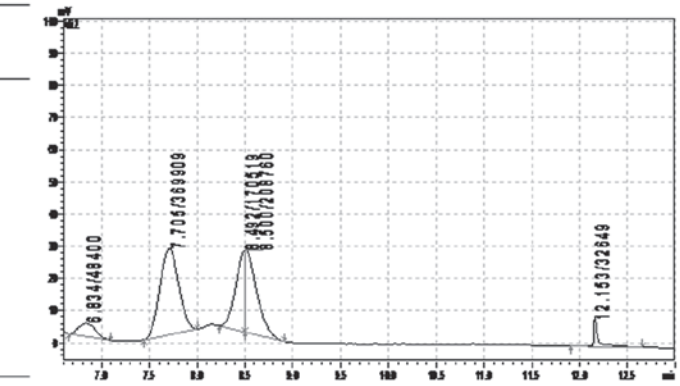


Figure 2. (A) Calibration curve of standard dextrans for CMN1 molecular weight determination. (B) Molecular weight of CMN1 was determined using a HPLC/ELSD system equipped with a TSK-GEL G4000PWXL column. (C) Retention time of the standard monosaccharide mixture (Rha; Xyl; Ara; Man; Glc; Gal). (D) Monosaccharide compositions of CMN1 were analyzed using an HPLC/ELSD system. HPLC, high performance liquid chromatography; ELSD, evaporative light scattering detector.

acid yield (0.06 mol/sugar residue, less than 0.1 mol/sugar residue) suggested that the 1→6 linkage in CMN1 was unimportant. In addition, the 1→2 linkages may have been the main chain linkage of CMN1, which was indicated by the high-yield glycerol production. A trace quantity of erythritol revealed the low content of 1→4 linkages in the main chain or branch. Collectively, the backbone of CMN1 was found to be composed of (1→2) linkages and (1→3) linkages with branched (1→6) linkages and (1→4) linkages.

The normobarie hypoxia, sodium nitrite toxicosis and acute cerebral ischemic-hypoxic tests (24) were used to detect the anti-hypoxic activity of CMN1. Compared with the NC group, CMN1 markedly improved the survival rate in the sodium nitrite toxicosis and normobarie hypoxia tests. Enhanced gasping persistence was also observed in the acute cerebral ischemic-hypoxic models. Additionally, water extracts from *Cordyceps sinensis* have been found to exhibit a scavenging effect on reactive oxygen species, superoxide

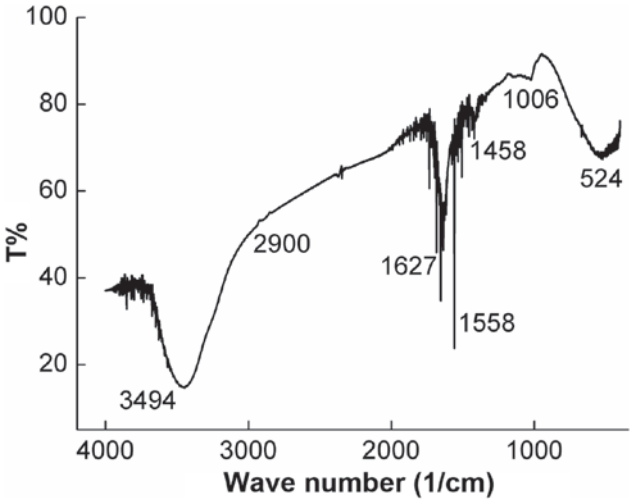
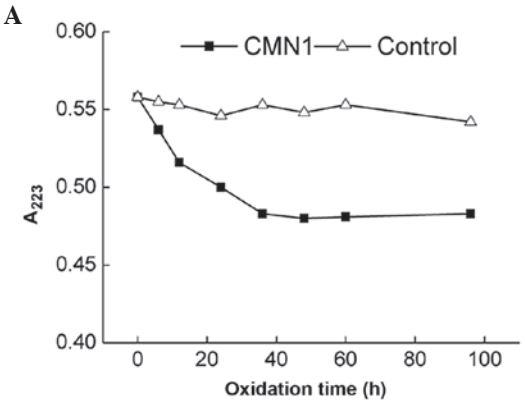


Figure 3. Fourier transform infrared spectroscopy spectrum of CMN1. T, transmittance.



B

The HPLC analysis of Smith degradation products

	Rha	Ara	Man	Gal	Glycerol	Erythritol
Before hydrolysis	+	+	–	–	–	–
After hydrolysis	–	+	–	–	+	+

"+"Detected;"–"Undetected

Figure 4. (A) Periodate oxidation time course. (B) Products of Smith degradation of CMN1 were detected using HPLC. HPLC, high performance liquid chromatography; Rha, L-rhamnose, Ara, L-arabinose; Man, D-mannose; Gal, D-galactose.

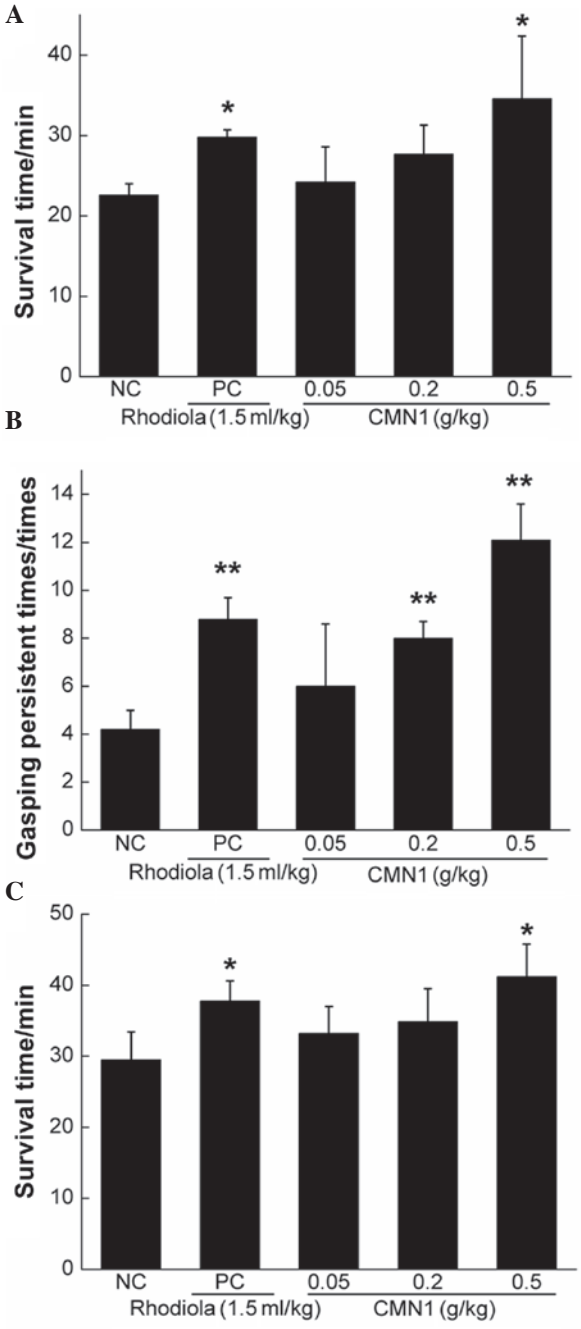


Figure 5. Anti-hypoxic effect of CMN1 determined using (A) sodium nitrite toxicosis, (B) acute cerebral ischemic-hypoxic and (C) normobarie hypoxia tests, respectively. The normobarie hypoxia test was performed in a sealed wide-mouth bottle containing soda lime. The sodium nitrite toxicosis test was performed by injection of 2 g/ml sodium nitrite. The acute cerebral ischemic-hypoxic test was performed by decapitation of the mice. Data are expressed as the mean ± standard deviation (n=10/group) and analyzed using one-way analysis of variance. *P<0.05 and **P<0.01, vs. the NC group. NC, normal control; PC, positive control.

anions and hydroxyl radicals by inhibiting malondialdehyde formation (33). Overall, the polysaccharide extracted from *Cordyceps militaris* was also effective against hypoxia.

Furthermore, the anti-hypoxic activity of CMN1 may be associated with its characteristic structure. CMN1 was observed to have a molecular weight of 37,842 Da and its monosaccharide composition consisted of Rha, Ara, Man and Gal. Compared with previous studies, CMN1 exhibits a similar primary structure (14), however, additional studies are required to identify the pharmacological efficacy and characteristic structures of the polysaccharides.

The present study was limited to one polysaccharide-enriched fraction separated from *Cordyceps militaris*. Whether other components exhibit significant roles in hypoxia remains to be elucidated. Therefore, further investigations focusing on *Cordyceps militaris* are required.

Acknowledgements

This study was supported by the twelfth Five-Year National key Technology R&D Program (grant no. 2012BAI29B00).

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