Carbohydrate Polymers 110 (2014) 405–414

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Chain conformation and immunomodulatory activity of a hyperbranched polysaccharide from *Cordyceps sinensis*

Ding-Tao Wu [1], Lan-Zhen Meng [1], Lan-Ying Wang [1], Guang-Ping Lv, Kit-Leong Cheong, De-Jun Hu, Jia Guan, Jing Zhao *, Shao-Ping Li *

State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 10 April 2014
Accepted 11 April 2014
Available online 21 April 2014

Keywords:
Cordyceps sinensis
Polysaccharide
Molecular mass
Chain conformation
Laser light scattering
Immunomodulatory activity

ABSTRACT

A polysaccharide, named as cordysinan, extracted from natural *Cordyceps sinensis*, was identified as a hyperbranched heteropolysaccharide from the results of FT-IR, GC–MS, and carbohydrate analysis by carbohydrate gel electrophoresis analysis, as well as the degree of branching of cordysinan was 43.3%. The solution properties of cordysinan were investigated by using size exclusion chromatography coupled with multi-angle laser light scattering and triple detector array, respectively. The molecular weights, the radius of gyration and the intrinsic viscosity of cordysinan were determined as 22.45 ± 0.26 kDa and 22.37 kDa, 15.4 ± 2.4 nm and 1.41 mL/g, respectively. By applying the polymer solution theory, the exponent (ν and α) values of $\langle S^2 \rangle_z^{1/2} = kM_w^{\nu}$ and $[\eta] = kM_w^{\alpha}$ were calculated as 0.28 and 0.42, respectively, which firstly revealed that cordysinan existed as a globular shape in 0.9% NaCl aqueous solution. Moreover, the results showed that cordysinan could obviously stimulate macrophages functions.

1. Introduction

Cordyceps, one of the well-known folk tonic foods and traditional Chinese medicines, is a composite consisting of the stromata of the fungus, *Cordyceps sinensis* (Berk.) Sacc. (Family: Hypocreaceae), which has been renamed as *Ophiocordyceps sinensis*, parasitized on the larva and the dead caterpillar. *Cordyceps* is commonly used in China and other Asian countries for prevention and treatment of a variety of diseases (Zhu, Halpern, & Jones, 1998a; Zhu, Halpern, & Jones, 1998b). *C. sinensis* contains high amount of polysaccharides, which could be ranged from 3 to 8% of the total dry weight (Li et al., 2006). Indeed, polysaccharides from *C. sinensis* are responsible for lots of bioactivities of *Cordyceps*, such as anti-tumor, immunomodulatory, anti-oxidation, hypoglycemic, and hypolipidemic activities (Ji et al., 2011; Li, Li, Dong, & Tsim, 2001; Ng and Wang, 2005; Yu, Wang, Huang, & Duh, 2006). Especially, the bioactivities of polysaccharides are closely correlated to their chemical properties such as molecular size, types and ratios of constituent monosaccharides, and features of glycosidic linkages (Hu, Cheong, Zhao, & Li, 2013), as well as the chain conformations (Li, Wu, Lv, & Zhao, 2013; Yang & Zhang, 2009). Up to date, various different polysaccharides from cultured *C. sinensis* have been extensively investigated, including the physico-chemical structures and the pharmacological functions (Yan, Wang, Li, & Wu, 2011; Zhong et al., 2009; Zhou, Gong, Su, Lin, & Tang, 2009). However, the structural features and the solution properties of polysaccharides from natural *C. sinensis* have seldom been reported although their pharmacological functions are widely studied (Zhou et al., 2009). Therefore, it is significant to investigate the fundamental information on physico-chemical structures of polysaccharides from natural *C. sinensis*, which is greatly helpful for revealing their structure–bioactivity relationship, and beneficial for development of polysaccharides from cultured *Cordyceps* as substitutes of polysaccharides from natural *C. sinensis*, as well as improving their quality control.

High performance size exclusion chromatography coupled with multi-angle laser light scattering (HPSEC-MALLS), a technique for determining, independently, the absolute molecular mass, the molecular mass distributions, and the radius of gyration, as well as

Abbreviations: ELSD, evaporative light-scattering detector; FPLC, fast protein liquid chromatography; FT-IR, Fourier transform-infrared spectroscopy; GC–MS, gas chromatography–mass spectrometry; HPSEC, high performance size exclusion chromatography; PACE, carbohydrate analysis by carbohydrate gel electrophoresis; RID, refractive index detection; SEC-MALLS, size exclusion chromatography coupled to Multi-angle laser light scattering; SEC-TDA, size exclusion chromatography coupled to triple detector array (online laser light scattering, refractometer, and viscometer).

* Corresponding authors. Tel.: +853 8397 4692; fax: +853 2884 1358.
E-mail addresses: zhaojing.cpu@163.com (J. Zhao), lishaoping@hotmail.com (S.-P. Li).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.carbpol.2014.04.044
0144-8617/

the direct chain conformation of polymers in solution, by detecting how they scatter light, is recognized as one of the most powerful techniques for the investigation of polymers (Li et al., 2013; Yang & Zhang, 2009; Zhang, Li, Wang, Zhang, & Cheung, 2011). Furthermore, HPSEC equipped with triple detector array (HPSEC-TDA) is a recently described and powerful technique for the characterization of macromolecules (La Gatta, De Rosa, Marzaioli, Busico, & Schiraldi, 2010; Piazza, Bertini, & Milany, 2010). The simultaneous determination of the absolute molecular mass, the dispersibility index, the radius of gyration, and the intrinsic viscosity allows the determination of a complete hydrodynamic characterization of a polymer in a single chromatographic analysis. Therefore, in the current study, HPSEC-TDA and HPSEC-MALLS techniques were performed for analyzing the molecular conformation of the polysaccharide. Furthermore, the murine macrophage cell line RAW 264.7 was used to investigate its immunomodulatory activity. This work for the first time provided fundamental information on the structure and the solution properties of polysaccharide from natural *C. sinensis*.

2. Materials and methods

2.1. Materials and chemicals

Natural *Cordyceps sinensis* were collected from Sichuan province, China. Identities of these samples were confirmed by Professor Shao-Ping Li, University of Macau, Macau SAR, China. The voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Monosaccharides standards, griess reagent, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and fluorescein isothiocyanate-conjugated dextran (FITC-Dextran 40,000) were purchased from Sigma (St. Louis, MO, USA). Laminaribiose, laminarihexaose, galactomannan, oat glucan, pectic galactan, β-glucanase (EC 3.2.1.6), dextranase (EC 3.2.1.11), α-amylase (EC 3.2.1.1), pectinase (EC 3.2.1.15) and β-mannanase (EC 3.2.1.78) were purchased from Megazyme (Wicklow, Ireland). Sephacryl S-200HR gel was purchased from GE Healthcare Co. 8-Aminonaphthalene-1,3,6 trisulphonic acid (ANTS) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Polyacrylamide containing a ratio of acrylamide/N,N-methylenebisacrylamide (19:1) was purchased from Bio-Rad (Hercules, CA, USA). Dulbecco's modified eagle medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS) were purchased from Invitrogen Molecular Probes (Carlsbad, CA, USA). Milliplex map kit was from Merck Millipore (Billerica, MA). Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Bedford, MA). Other reagents were analytical grade.

2.2. Extraction and purification of polysaccharide

Two kilograms of natural *C. sinensis* were dried at 40 °C for 12 h and grounded to powder (100 mesh). The powder was extracted by supercritical fluid extraction (SFT-250 SFE/SFR system, Supercritical Fluid Technologies, INC, Newark, Delaware, USA). Subsequently, the remains were treated with petroleum ether and 70% ethanol to remove the lipophilic and low polar compounds, respectively. At last, the residues were suspended in 5-fold volume of deionized water and extracted at 100 °C for 1 h. The concentrated extractions were centrifuged at 3000 × *g* for 20 min (Allegra X-15R, Beckman Coulter, Fullerton, CA, USA), and the supernatant was transferred to a membrane ultrafiltration (molecular weight cutoff: 3 kDa, Millipore, Billerica, MA, USA), and then the low molecular weight compounds (Mw < 3 kDa) were removed. Then the solutions were collected and freeze-dried using a freeze dryer (Thermo Savant YOD-230, Labconco, USA) to yield dried powder of crude polysaccharides.

Furthermore, the stepwise ethanol precipitation was performed. The powder of crude polysaccharides was dissolved in deionized water, and ethanol was added to final concentrations of 20%, 30%, 40%, 50%, 60% and 75% (v/v), respectively. After standing for 12 h at 4 °C, centrifugation (4500 × *g* for 15 min) was performed. The precipitate was dried on water bath (60 °C), and then redissolved in hot water (60 °C) for 30 min. After centrifugation (4500 × *g* for 15 min), the powder of the supernatant was obtained by freeze-drying.

Fast protein liquid chromatography (ÄKTA explorer purification system, GE Healthcares, Uppsala, Sweden) was used for purification of polysaccharides. One of the dried powder (60% precipitated fraction) was dissolved in 0.2 M ammonium carbonate solution (pH 8.7), then the supernatant was loaded on a Sephacryl S-200HR gel column (100 cm × 1.6 cm, i.d.) and was eluted with 0.2 M ammonium carbonate solution (pH 8.7) at a flow rate of 0.5 mL/min. The procedure was monitored by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the homogeneity of the fractions was also detected by high performance size exclusion chromatography coupled with diode array detector followed by evaporative light-scattering detector (HPSEC-DAD/ELSD) as previous reported methods with minor modification (Guan, Zhao, Feng, Hu, & Li, 2011). Briefly, HPSEC-DAD/ELSD measurements were carried out on an Agilent 1100 series liquid chromatography (LC)–DAD system (Agilent Technologies, Palo Alto, CA, USA) equipped with a column of TSK-GEL G-3000PW$_{XL}$ (300 mm × 7.8 mm, i.d., Tosoh Bioscience, Tokyo, Japan) at 35 °C. An ELSD detector (Alltech ELSD 2000ES, Grace, IL, USA) was simultaneously connected. Finally, the purified polysaccharides named as cordysinan were obtained.

2.3. Chemical structure characterization

2.3.1. FT-IR spectra analysis

1 mg of cordysinan was mixed with 200 mg of dried KBr, and pressed into disk for the analysis. The IR spectra were recorded in the frequency range of 4000–450 cm^{-1} with a Spectrum 100 FT-IR (PerkinElmer, Waltham, MA, USA) instrument, and the resolution was set as 4 cm^{-1}.

2.3.2. GC–MS analysis

Analysis of compositional monosaccharide: The sample of cordysinan (3 mg) was hydrolyzed with 2 M trifluoroacetic acid (3 mL) at 95 °C in a sealed tube for 12 h. After hydrolysis, the hydrolysates were washed with methanol and evaporated to dryness with a nitrogen evaporator for three times to remove the residue of trifluoroacetic acid. Subsequently, 0.5 mL of pyridine and 10 mg of hydroxylamine hydrochloride were added and incubated at 90 °C for 30 min, then 0.5 mL of acetic anhydride was added and incubated at 90 °C for 30 min. The derivatives were analyzed by using an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA). A capillary column (30 m × 0.25 mm, i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. High purity helium was used as carried gas with a flow rate of 1 mL/min. The column temperature was set at 165 °C and held for 7 min for injection, then programmed at 5 °C/min to 185 °C and held for 5 min, then at 4 °C/min to 200 °C, and finally, at 20 °C/min to 280 °C, and held for 2 min. The split ratio was set as 10:1. The spectrometers were operated in electron impact mode, the ionization energy was 70 eV and the scan rate was 0.34 s per scan. The temperatures of ionization source and the transfer line were 150 °C and 280 °C, respectively.

Methylation analysis of cordysinan under microwave irradiation: The methylation was carried out according to the reported method

with minor modification (Singh & Tiwari, 2008). Briefly, to a solution of cordysinan (3 mg in 2 mL of DMSO with 20 mg of NaOH), 0.15 mL of CH_3I was added. After each addition of the reagents, the reaction mixture was performed under microwave irradiation (Multiwave 3000, Anton paar GmbH, Graz, Austria). The microwave irradiation program was performed at 500 W for 30 s. Finally the reaction mixture was cooled to room temperature and dialyzed overnight against deionized water (molecular weight cut-off: 3.5 kDa), then the reaction mixture was evaporated to dryness with a nitrogen evaporator. The methylated cordysinan was dissolved in 2 mL of 90% HCOOH, and the solution was exposed to microwave irradiation under 400 W and 100 °C for 5.5 min, then the solution was evaporated to dryness with a nitrogen evaporator. Subsequently, the residue was redissolved in 2 mL of 2 M TFA and exposed to microwave irradiation under 400 W and 100 °C for 5.5 min. Finally, the partially methylated monosaccharides were converted to the partially methylated alditol acetates according to the reported method (Hakomori, 1964). Briefly, the partially methylated monosaccharides were reduced with $NaBH_4$, and neutralized with acetic acid, then acetylated with acetic anhydride. GC–MS was performed under the above mentioned conditions with minor modification. Briefly, the column temperature was set at 120 °C for injection, then programmed at 5 °C/min to 200 °C, and finally, 8 °C/min to 250 °C.

2.3.3. Saccharide mapping based on PACE analysis

PACE was performed to characterize the structures of cordysinan according to our previously reported method (Wu, Xie, Hu, Zhao, & Li, 2013), which was described as follows.

Cordysinan solutions (5 mg/mL, 100 μL) were mixed with certain enzymes (the final concentration of β-D-glucanase, β-D-mannanase, pectinase, dextranase and α-amylase were 2 U/mL, 2 U/mL, 20 U/mL, 2 U/mL and 20 U/mL, respectively) in a total volume of 1000 μL and digested overnight (12 h) at 40 °C. Then the mixtures were heated at 80 °C for 20 min to stop the enzymatic digestion, and evaporated to dryness with a nitrogen evaporator at 40 °C. Cordysinan solution without enzymes, treated as described above, was used as blank control. The polysaccharide standards, oat glucan, galactomannan, pectic galactan, dextran and soluble-starch (5 mg/mL, 100 μL), were treated with corresponding enzymes, respectively, under the above conditions. Enzymatic digestions of cordysinan were derivatized with ANTS at 37 °C for 17 h. The derivatized sugars were resuspended in 0.5 mL of urea (6 mol/L) solution. All samples were separated using a vertical slab gel electrophoresis apparatus, Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA), with 10 cm plates, 1.0-mm spacer, and well of width 0.3 cm. Electrophoresis of 30% (w/v) polyacrylamide in the resolving gel with a stacking gel of 8% (w/v) polyacrylamide was used for separation of enzymatic hydrolysates. The electrophoresis buffer was 0.1 mol/L pH 8.2 Tris–boric acid. The samples were electrophoresed first at 200 V for 20 min and then at 700 V for 45 min, to move bromophenol blue (migration indicator) to the designed level. All runs were performed at least two times independently. Gels were imaged using an InGenius LHR CCD camera system (Syngene, Cambridge, UK) under UV 365 nm.

2.4. Characterization of chain conformation

2.4.1. Determination of the differential refractive index increment (dn/dc)

The specific refractive index increment (dn/dc) of cordysinan in 0.9% aqueous NaCl solution were measured by using an Optilab rEX refractometer (DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA) at 658 nm and 35 °C. Cordysinan in 0.9% NaCl aqueous solution at various concentrations ranges from 0.2 mg/mL to 1.0 mg/mL was measured by RI batch model. All of the polysaccharide solutions were purified by a 0.2 μm filter before use.

2.4.2. Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS)

The values of weight-average molecular weight (M_w) and radius of gyration ($\langle S^2 \rangle_z^{1/2}$) of cordysinan in 0.9% NaCl aqueous solution were determined by using SEC-MALLS. SEC-MALLS measurements were carried out on a multi-angle laser light scattering (DAWN HELEOS, Wyatt Technology Co., Santa Barbara, CA, USA) with an Agilent 1100 series high performance liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a column of TSK-GEL G-4000PW$_{XL}$ (300 mm × 7.8 mm, i.d., Tosoh Bioscience, Tokyo, Japan) at 35 °C. An Optilab rEX refractometer (DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA) was simultaneously connected. The MALLS instrument was equipped with a He–Ne laser (λ = 658 nm) at angles (θ) of 60, 69, 80, 90, 100, 111, 121, 132, 149, 165° at 35 °C. The M_w and $\langle S^2 \rangle_z^{1/2}$ were calculated by the Zimm method of static light scattering based on the basic light scattering equation is as follows:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{16\pi^2 \langle S^2 \rangle_z}{3\lambda^2} \sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C + \cdots \quad (1)$$

where K was an optical constant equal to $[4\pi^2 n^2 (dn/dc)^2]/(N_A \lambda^4)$; C, the polysaccharide concentration; R_θ, the Rayleigh ratio; λ, the wavelength; n, the refractive index of the solvent (0.9% NaCl aqueous solution); dn/dc, the refractive index increment of cordysinan in 0.9% NaCl aqueous solution; N_A, the Avogadro's number; A_2, the second virial coefficient. The mobile phase was 0.9% NaCl aqueous solution at a flow rate of 0.5 mL/min. All of the polysaccharide solutions were purified by a 0.2 μm filter before use. The injection volume was 100 μL with a concentration of 1 mg/mL for the sample. The Astra software (version 6.0.2, Wyatt Tech. Corp.) was utilized for data acquisition and analysis.

2.4.3. Size exclusion chromatography coupled to triple detector array (SEC-TDA)

The values of weight-average molecular weight (M_w), radius of gyration ($\langle S^2 \rangle_z^{1/2}$) and intrinsic viscosity ($[\eta]$) of cordysinan in 0.9% NaCl aqueous solution were also determined by using SEC-TDA (Viscotek, Malvern, UK). SEC-TDA measurements were carried out on a Viscotek system. The Agilent 1200 series high performance liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a column of TSK-GEL G-4000PW$_{XL}$ (300 mm × 7.8 mm, i.d., Tosoh Bioscience, Tokyo, Japan) was coupled with a Viscotek mod.302 Triple Detector Array. The triple detector array consisted of a refractive index detector, a four-bridge viscometer, and a light scattering detector. The latter consisted of a right angle laser light scattering and a low angle (7°) laser light scattering equipped with a He–Ne laser (λ = 670 nm). The M_w was calculated by the Zimm method of static light scattering based on the basic light scattering equation is as follows:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P_\theta} + 2A_2C + \cdots \quad (2)$$

where K was an optical constant equal to $[4\pi^2 n^2 (dn/dc)^2]/(N_A \lambda^4)$; C, the polysaccharide concentration; R_θ, the Rayleigh ratio; λ, the wavelength; n, the refractive index of the solvent (0.9% NaCl aqueous solution); dn/dc, the refractive index increment of cordysinan in 0.9% NaCl aqueous solution; N_A, the Avogadro's number; A_2, the second virial coefficient. P_θ, the particle scattering factor, the value is about 1 at low angle (7°). Moreover, the intrinsic viscosity was

calculated by using the Solomon-Ciută approximation (Solomon and Ciută, 1962),

$$[\eta] = \frac{\left[2(\eta_{sp} - \ln(\eta_{sp} + 1))\right]^{0.5}}{C} \quad (3)$$

where η_{sp} is the specific viscosity equal to $(\eta - \eta_o)/\eta_o$, η, intrinsic viscosity of cordysinan, η_o, intrinsic viscosity of the solvent. C, the concentration of cordysinan; column, injector and detectors were maintained at 35 °C. An aqueous solution of 0.9% NaCl was used as mobile phase at a flow rate of 0.5 mL/min. The system was calibrated with the polyethylene oxide narrow and dextran broad standards of known M_w, polydispersity and intrinsic viscosity. All of the polysaccharide solutions were purified by a 0.2 μm filter before use. The injection volume was 100 μL with a concentration of 1 mg/mL for the sample. The OmniSEC software (version, 4.7.0) was used for the acquisition and analysis of the viscotek data of samples.

2.5. Effects of cordysinan on the macrophages functions

2.5.1. Cell culture

RAW 264.7 mouse macrophage cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS, 1% Penicillin/Streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.5.2. Cytotoxicity assay

RAW 264.7 cells (5×10^3 cells/well) were cultured in 96-well microplates overnight, and then treated with serial concentrations of cordysinan and LPS (0.4 μg/mL) for 24 h, respectively. Equal volume of culture medium was used as blank control. Subsequently, cells were stained with MTT at final concentration of 0.5 mg/mL in PBS (pH 7.4) for 4 h in dark and then the medium was discarded. The formazan crystals presented in cells were dissolved by 100 μL of DMSO. The absorbance was read at 570 nm on a microplate reader (1420 Multilabel counter victor3, Perkin-Elmer, MA). The results were expressed as ratio of absorbance values between treatment and blank control.

2.5.3. Nitric oxide assay

RAW 264.7 cells (5×10^4/well) were seeded in 96-well microplates overnight, and then were stimulated by serial concentrations of cordysinan and LPS (0.4 μg/mL) for 18 h, respectively. Equal volume of culture medium was used as blank control. Subsequently, 75 μL of supernatants were collected and mixed with an equal volume of modified Griess reagent at room temperature for 15 min. The absorbance was measured at 540 nm with microplate reader. The NO production was expressed as ratio of absorbance values between treatment groups and LPS treated-group.

2.5.4. Pinocytosis assay

RAW 264.7 cells (20×10^4/well) were plated in 24-well plates overnight, and then exposed to serial concentrations of cordysinan for 18 h. Equal volume of LPS (0.4 μg/mL) and culture medium were used as the positive and blank control, respectively. Then the cells were treated with FITC-dextran (0.1 mg/mL) and incubated for 60 min at 37 °C with 5% CO_2. After incubation, cells were washed for four times in cold PBS contained 2% FBS, and analyzed by flow cytometry (BD Biosciences, San Jose, CA). The pinocytic ability was shown as mean fluorescence intensity (MFI)-FITC of macrophages (BD FACSDiva Software V6.1.3). The results were expressed as ratio of pinocytic activity between treatment and blank control.

2.5.5. Quantitative analysis of cytokines

RAW 264.7 cells (5×10^4/well) were seeded in 96-well plates overnight and then exposed to serial concentrations of cordysinan and LPS (0.4 μg/mL) for 24 h, respectively. The cell supernatants were collected by centrifugation at 1000 × g for 10 min. The levels (pg/mL) of cytokines/chemokines in culture supernatant were measured by using a Luminex assay (Bio-Plex™ 200, Hercules, CA) with commercially available Millilex Map kits according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager™ software 5.0 (Bio-Rad).

2.5.6. Determination of endotoxin contamination

The endotoxin concentration in the cordysinan were tested by using Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD) with a Glucashield reconstitution buffer formulated to block interference of 1,3-β-D-glucans (Associates of Cape Cod). Briefly, the concentration of endotoxin in the test samples were calculated from the absorbance values of solutions containing known amounts of endotoxin standard (*Escherichia coli* O113:H10 endotoxin, Associates of Cape Cod, Falmouth, MA). The results indicated that cordysinan contained less than 0.1 ng endotoxin per 1 μg. Therefore, the possibility of endotoxin contamination in cordysinan could be excluded.

2.6. Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed using a two-tailed Student's t test (GraphPad Prism 5.0), and statistical differences were considered significant if the p value was <0.05.

3. Results and discussion

3.1. Chemical structure characterization of cordysinan

The yield of crude polysaccharide from *C. sinensis* was 3.75%. The crude polysaccharides were purified by stepwise ethanol precipitation and Sephacryl S-200HR chromatography. For the fractionation of the 60% fraction, which exhibited significant immunomodulatory activity, a single peak was obtained after elution on the Sephacryl S-200HR (Fig. 1A). The results of HPSEC-MALLS chromatogram (Fig. 1B) and HPSEC-DAD/ELSD chromatogram (Fig. 1C) indicated that the purified polysaccharide, named as cordysinan, was a homogeneous polysaccharide. Additionally, the total sugar content of cordysinan was determined as 92.06% by the phenol-sulfuric acid method.

The IR spectrum of cordysinan (Fig. 2A) exhibited a strong band at 3410.2 cm^{-1}, attributing to the hydroxyl stretching vibration. The peak at 2928.5 cm^{-1} was due to C—H stretching vibration, and the absorption peak around 1647.6 cm^{-1} was due to associated water. The broad absorption bands at 1548.2, 1402.3, and 1231.5 cm^{-1}, could be assigned to C—H deformation vibration and C—OH bending vibrations (Xu, Chen, Wang, & Zhang, 2009; Xu, Chen, Zhang, & Ashida, 2012). The absorption peak around 1231.5 cm^{-1} was assigned to C—O stretching in the ring. Peak at 1402.3 cm^{-1} was from a CH_2 vibration. The peak at 817.1 cm^{-1} was assigned to α-D-galactose according to the previous studies (Wang, Peng, et al., 2011; Yang & Zhang, 2009).

Compositional monosaccharides of cordysinan were determined by TFA hydrolysis and GC–MS analysis (data unshown). The results showed that Man, Gal and Glc were the major monosaccharides of cordysinan with a molar ratio of 4.4:3.8:1.0, which were accordance with our previous results that compositional monosaccharides of crude polysaccharide in natural *Cordyceps* were Man, Glc and Gal (Guan, Yang, & Li, 2010). However, the major compositional monosaccharides of polysaccharides from most of the

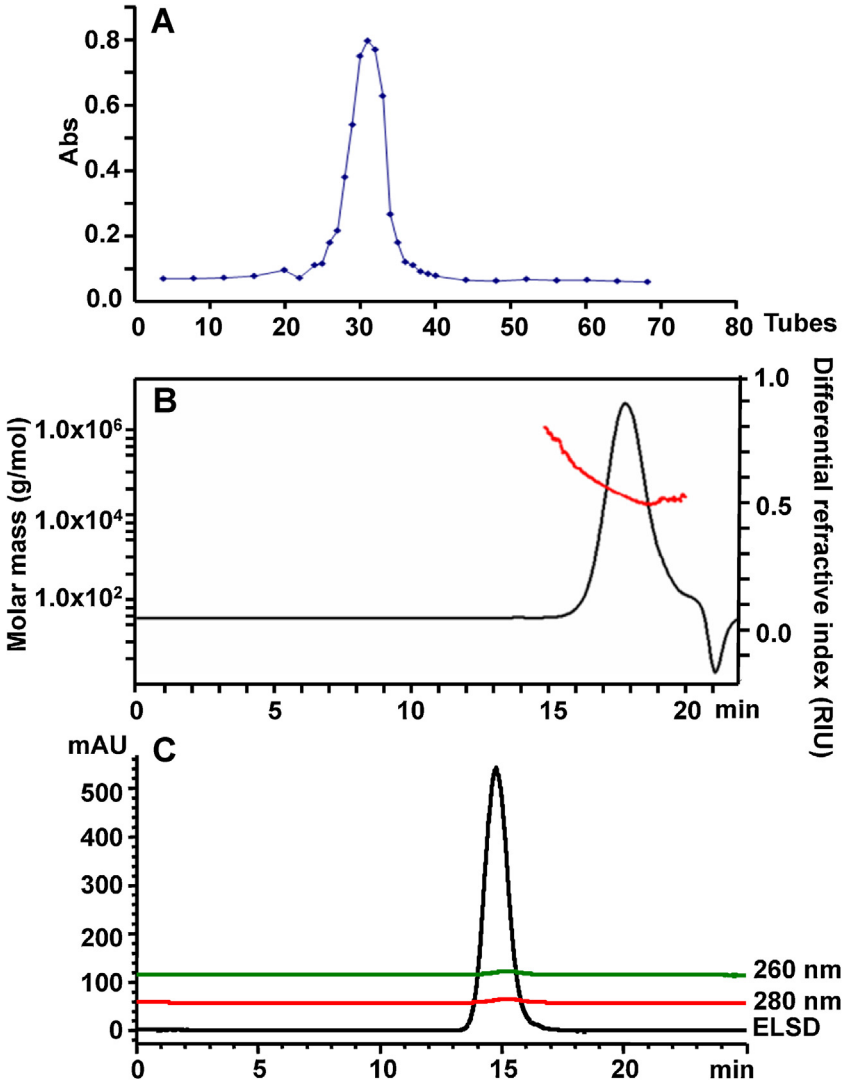

Fig. 1. The gel permeation (A), HPSEC-MALLS (B) and HPSEC-DAD/ELSD (C) chromatograms of cordysinan from natural *C. sinensis*.

cultural *C. sinensis* were glucose (Nie, Cui, Xie, Phillips, & Phillips, 2013; Nie et al., 2011; Wang, Peng, et al., 2011; Wu, Sun, & Pan, 2005; Wu, Sun, & Pan, 2006; Yan et al., 2011), which were different from those of natural *C. sinensis*.

Methylation of cordysinan was performed under the microwave irradiation conditions, which has been proved as a powerful approach for the grafting modification of polysaccharides (Singh, Kumar, & Sanghi, 2012; Singh & Tiwari, 2008). The method is efficient and less time consuming, particularly, inert atmosphere is not required for the reaction. Table 1 summarizes the experimental results of methylated hydrolysates of cordysinan on GC–MS. The results showed the presence of twelve components, namely 2,3,4,6-Me_4-Man, 2,3,4,6-Me_4-Glc, 2,3,4,6-Me_4-Gal, 3,4,6-Me_3-Man, 2,3,6-Me_3-Man, 2,3,6-Me_3-Glc, 2,3,4-Me_3-Gal,

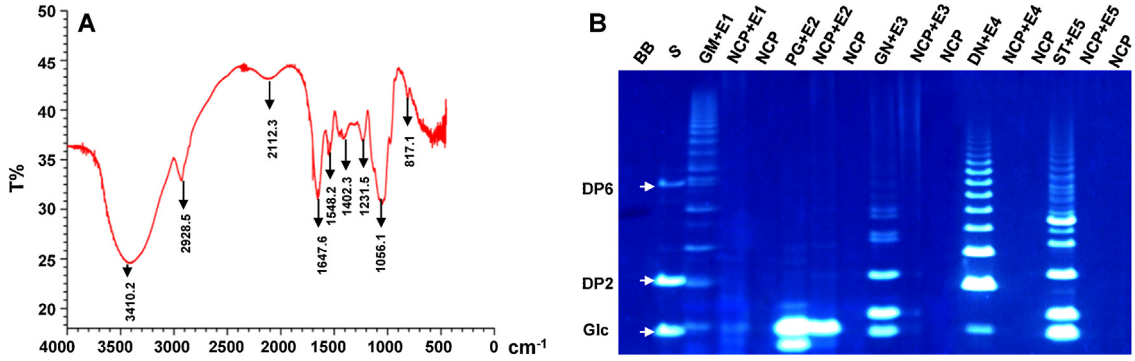

Fig. 2. Infrared spectra (A) and PACE profile of enzymatic digestions (B) of cordysinan from natural *C. sinensis*. BB, bromophenol blue; S, mixture of glucose, laminariose (DP2), and laminarihexaose (DP6); NCP, cordysinan without enzyme; GM, PG, GN, DN and ST, hydrolysates of galactomannan, pectic galactan, β-1,3(4)-glucan, dextran and soluble-starch, respectively; E1–E5, 1,4-β-mannanase, pectinase, 1,3(4)-β-glucanase, dextranase and α-amylase, respectively;

Table 1
GC–MS results of partially methylated alditol acetates of cordysinan from natural *C. sinensis*.

Signals	RT (min) [a]	Methylated sugar	Linkage type	Molar ratios
1	1.000	1,5-O-Ac_2-2,3,4,6-Me_4-mannitol[b]	T-Man*p*	2.1
2	1.096	1,5-O-Ac_2-2,3,4,6-Me_4-glucoitol	T-Glc*p*	6.4
3	1.107	1,5-O-Ac_2-2,3,4,6-Me_4-galactitol	T-Gal*p*	3.1
4	1.241	1,2,5-O-Ac_3-3,4,6-Me_3-mannitol	1,2-Man*p*	18.9
5	1.252	1,4,5-O-Ac_3-2,3,6-Me_3-mannitol	1,4-Man*p*	2.7
6	1.264	1,4,5-O-Ac_3-2,3,6-Me_3-glucoitol	1,4-Glc*p*	2.3
7	1.293	1,5,6-O-Ac_3-2,3,4-Me_3-galactitol	1,6-Gal*p*	5.5
8	1.332	1,4,5-O-Ac_3-2,3,6-Me_3-galactitol	1,4-Gal*p*	1.2
9	1.367	1,2,3,5-O-Ac_4-4,6-Me_2-mannitol	1,2,3-Man*p*	3.8
10	1.434	1,2,5,6-O-Ac_4-3,4-Me_2-galactitol	1,2,6-Gal*p*	3.9
11	1.452	1,4,5,6-O-Ac_4-2,3-Me_2-galactitol	1,4,6-Gal*p*	1.0
12	1.464	1,2,3,4,5-O-Ac_5-6-Me-mannitol	1,2,3,4-Man*p*	1.3

[a] Retention time (RT) of sugars relative to that of 1,5-O-Ac2-2,3,4,6-Me4-mannitol.
[b] 1,5-O-Ac_2-2,3,4,6-Me_4-mannitol = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-mannitol.

2,3,6-Me_3-Gal, 4,6-Me_2-Man, 3,4-Me_2-Gal, 2,3-Me_2-Gal, and 6-Me-Man in a molar ratio of 2.1:6.3:3.1:18.9:2.7:2.3:5.5:1.2:3.8:3.9:1.0:1.3 (about 2:6:3:19:3:2:5:1:4:4:1:1), which indicated that the cordysinan had a main backbone of 1,2-Man*p* residues. Additionally, the results showed a good correlation between terminal and branched residues. These results are helpful for quality control of polysaccharides in natural *C. sinensis*. The degree of branching (DB) was calculated on the basis of the formula (Tao, Zhang, Yan, & Wu, 2007), $DB = (N_T + N_B)/(N_T + N_B + N_L)$, where N_T, N_B, N_L are the total numbers of the terminal residues, branched residues, and linear residues, respectively. Then the DB value of cordysinan was calculated to be 43.3%, indicating that cordysinan was a hyperbranched heteropolysaccharide, which were different from the polysaccharides from cultural *C. sinensis* (Nie et al., 2013). Usually, the hyperbranched polymers possess specific properties, such as low viscosity, high solubility, large numbers of functional end groups, and roughly globular shape in solution, which are beneficial for its application in biology and pharmacology (Yang et al., 2010).

Furthermore, the method of saccharide mapping was performed for characterization of cordysinan. In our previous studies, the crude polysaccharides from natural *C. sinensis* contained 1,4-β-D-glucosidic, 1,4-β-D-mannosidic, 1,6-α-glucosidic, 1,4-α-glucosidic and 1,4-α-galactosidic linkages (Guan et al., 2011). Also based on the results of methylation analysis of cordysinan, the endo-carbohydrase including β-D-glucanase, β-D-mannanase, pectinase, dextranase and α-amylase were selected for hydrolysis of cordysinan. As shown in Fig. 2B, cordysinan could be digested to produce small sugars with β-D-glucanase, β-D-mannanase, and pectinase, respectively, which were not existed in samples before enzymatic hydrolysis. However, dextranase and α-amylase have no effect on the cordysinan (Fig. 2B), which suggested that α-1,6-glucosidic and α-1,4-glucosidic linkages might not be existed. The positive response of cordysinan to β-D-mannanase suggested that β-1,4-mannosidic linkages were existed, which was accordance with the results from methylation analysis. β-D-glucanase can act 1,4 or 1,3 bonds on the β-1,3(4)-D-glucan. Meanwhile, our previous studies showed that β-1,3-D-glucanase had no effect on the polysaccharide from *C. sinensis* (Guan et al., 2011). Therefore, the positive response of the cordysinan to β-D-glucanase mainly due to its β-1,4-glucosidic linkages, which was accordance with the results from methylation analysis. Additionally, the positive response of cordysinan to pectinase combined with its compositional monosaccharides suggested that α-1,4-galactosidic linkages were existed, which was accordance with the results from methylation analysis. These results are beneficial for development of polysaccharides from cultured *Cordyceps* as substitutes of polysaccharides from natural *C. sinensis* and improvement of their quality control.

3.2. Molecular parameters of cordysinan

In the batch mode of the RI experiment, the samples were measured by the Optilab rEX refractometer at dilute concentrations. Fig. 3A shows the plots of differential refractive index versus concentration for the cordysinan in 0.9% NaCl aqueous solution. The specific refractive index increment (dn/dc) of cordysinan was measured as 0.1365 mL/g.

SEC-MALLS, an absolute method, has been approved as the simple and efficient method for analysis of the absolute molecular mass (M_w), the dispersibility index (PDI), and the radius of gyration ($\langle S^2 \rangle_z^{1/2}$) in dilute polymer solution, as well as the direct chain conformation of polymers (Yang & Zhang, 2009; Zhang et al., 2011). The values of M_w and $\langle S^2 \rangle_z^{1/2}$ of cordysinan could be obtained directly by computing a classical Zimm polt from light scattering data collected at various angles (θ) for each concentration. Fig. 3B illustrates the angle dependences of $(Kc/R_\theta)c = 0$ for the cordysinan in 0.9% NaCl aqueous solution at 35 °C. The M_w, the PDI and the $\langle S^2 \rangle_z^{1/2}$ determined by SEC-MALLS were 22.45 ± 0.26 kDa, 1.2, and 15.4 ± 2.4 nm, respectively. Fig. 3C shows the SEC-MALLS chromatography and molecular mass distribution of cordysinan in 0.9% NaCl aqueous solution at 35 °C, which indicates that the cordysinan is the homogeneous polysaccharide with narrow molecular mass distribution. Furthermore, the relatively low $\langle S^2 \rangle_z^{1/2}$ value for cordysinan suggested that the polysaccharide molecules exist as compact conformation in aqueous solution (Huang, Huang, Li, & Zhang, 2009).

Additionally, the M_w, $\langle S^2 \rangle_z^{1/2}$ and intrinsic viscosity ($[\eta]$) were also determined by SEC-TDA. Fig. 3D shows the SEC-TDA chromatography and molecular mass distribution of cordysinan in 0.9% NaCl aqueous solution at 35 °C. The results also indicated that cordysinan was the homogeneous polysaccharide with narrow molecular mass distribution. The M_w, PDI, and $[\eta]$ of cordysinan measured by SEC-TDA were 22.37 kDa, 1.29, and 1.41 mL/g, respectively. The values of M_w and PDI were accordance with those of SEC-MALLS. However, the radius of gyration of cordysinan could not be detected due to the limit of detection of TDA. Furthermore, the relatively low intrinsic viscosity of cordysinan may attribute to its branched structures and low molecular weight. Actually, the hyperbranched polymers usually possess the high solubility and the low viscosity (Yang et al., 2010). The $[\eta]$ is an intrinsic property of the chain of polymer, which reflects the shape and volume of the polymer in the solvent. Usually, a lager value of $[\eta]$ reflects the extended rigid chain, and smaller value of $[\eta]$ indicates compact coil (Ma, Wang, & Zhang, 2008). For instance, the polysaccharides from *Rhizoma Panacis Japonici* had the M_w of 35 kDa and the $[\eta]$ of 7.9 mL/g in 0.15 M aqueous NaCl solution, which existed as

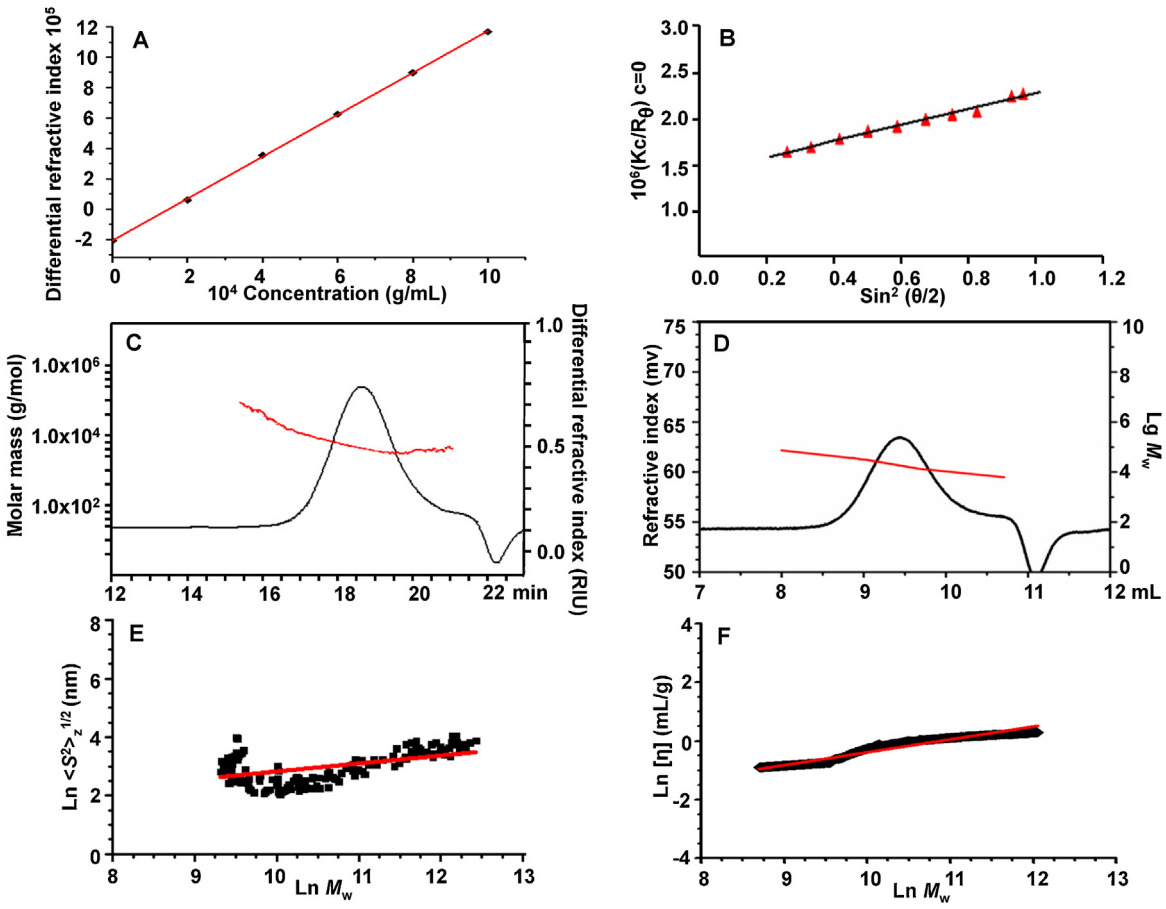

Fig. 3. Plots of differential refractive index versus concentration (A) and angular dependence of $(Kc/R_\theta)_{c=0}$ (B), SEC-MALLS (C) and SEC-TDA (D) chromatograms, and plots of $\left\langle S^2\right\rangle_z^{1/2}$ versus M_w (E) and $[\eta]$ versus M_w (F) in double logarithmic coordinates for cordysinan in 0.9% NaCl aqueous solution at 35 °C.

spherical chain conformation (Huang et al., 2009). Therefore, the lower $[\eta]$ of cordysinan in 0.9% aqueous NaCl solution may suggest that cordysinan exists as compact chain conformation.

3.3. Chain conformation of cordysinan

The bioactivities of polysaccharide from natural resources are strongly affected by their chain conformations (Li et al., 2013). For instance, the anti-tumor activities of the well known glucans (lentinan and schizophyllan) were mainly attributed to their triple helix chain conformations (Yang & Zhang, 2009; Zhang et al., 2011). Therefore, the investigation of the chain conformation of cordysinan from natural *C. sinensis* in aqueous solution is essential to correlate their advanced structures to their bioactivities. The conformations of polysaccharides can be analyzed based on the theory of dilute polymer solutions. Therefore, the chain conformation of cordysinan in 0.9% NaCl aqueous solution can be characterized based on the abovementioned data. The power law function $\left\langle S^2\right\rangle_z^{1/2} = kM_w^{\nu}$ can be estimated from many experimental points in the SEC-MALLS chromatogram. Fig. 3E shows the plots of $\left\langle S^2\right\rangle_z^{1/2}$ versus M_w for cordysinan in double logarithmic coordinates. The exponent (ν) of the power law function $\left\langle S^2\right\rangle_z^{1/2} = kM_w^{\nu}$ was calculated as 0.28. Generally, the ν exponents of 0.2–0.4 for branching polymers with compact coil chain conformation, 0.3 for sphere-like, 0.5–0.6 for a flexible polymer in a good solvent, and 0.6–1.0 for a semi-flexible chain (Kogan, Alföldi, & Masler, 1988; Wang, Ma, et al., 2011). In view of the data, cordysinan existed as a globular shape with branching. However, the power law function was more suitable for the determination of chain conformation of polysaccharides with the molecular weight and the radius of gyration more than 100 kDa and 10 nm, respectively. Therefore, the chain conformation of cordysinan should be further determined and confirmed by using SEC-TDA containing the viscometer detector.

The exponent (α) of the Mark–Houwink equation $[\eta] = kM_w^{\nu}$ is usually related to the shape and the nature of polymers in solution, which can be estimated by using SEC-TDA. Fig. 3F shows the plots of $[\eta]$ versus M_w for cordysinan in 0.9% NaCl aqueous solution at 35 °C in double logarithmic coordinates. The exponent (α) of the Mark–Houwink equation ($[\eta] = kM_w^{\alpha}$) was calculated as 0.42. In general, α value of 0.5 suggests that the polymer molecules exist as a global shape, and range from 0.6 to 0.8 indicated a flexible chain, above 0.8 and even beyond 1.0 for rigid polymer chains (Burchard, 1999; Scherrenberg et al., 1998; Yang & Zhang, 2009). Therefore, cordysinan was further confirmed as globular shape in 0.9% NaCl aqueous solution at 35 °C, which was similar with the chain conformation of the branched polysaccharides with anti-tumor activity from *Ganoderma lucidum* (Wang, Ma, et al., 2011) and *Pleurotus tuber-regium* (Tao, Zhang, & Zhang, 2009), respectively. However, the chain conformation of cordysinan was different from that of polysaccharides from cultural *C. sinensis* and other mushrooms, which formed as random coils (Yan et al., 2011; Yan, Wang, Ma, & Wu, 2013), and triple/double/single helix, flexible chain and stiff chain (Li et al., 2013; Yang & Zhang, 2009) in the aqueous solution, respectively. These results are important and helpful for investigation of the structure-activity relationship of polysaccharides from *C. sinensis*.

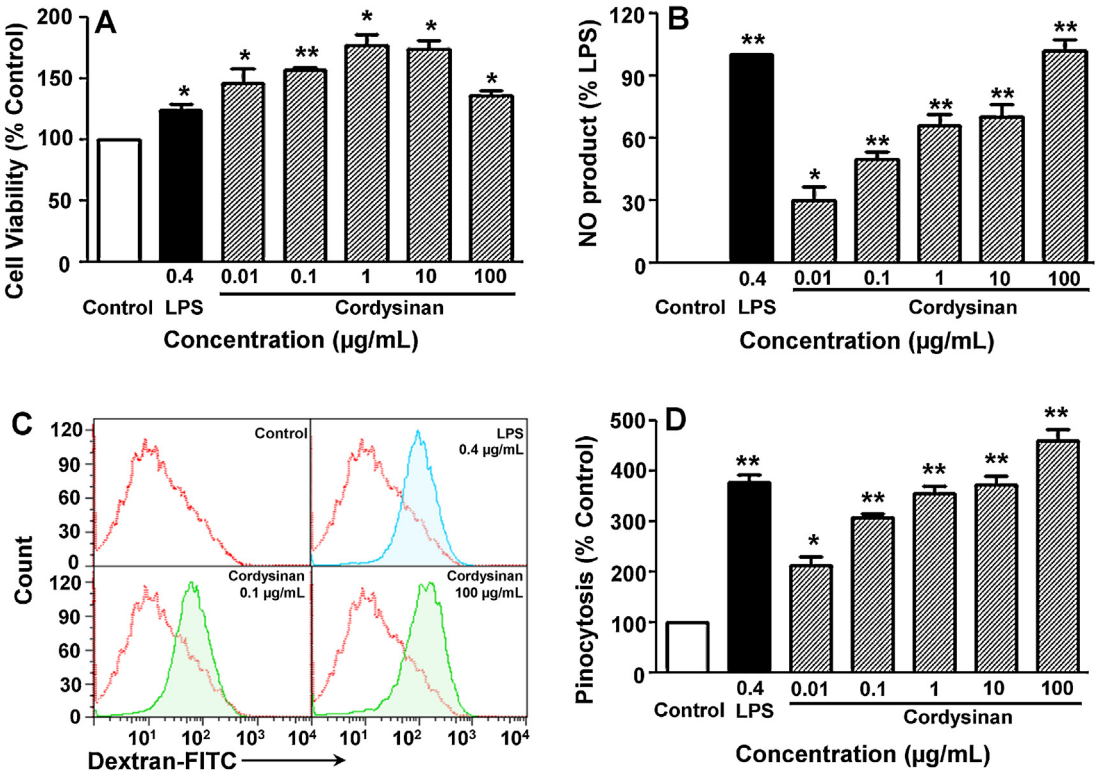

Fig. 4. Effects of cordysinan on RAW 264.7 cells A, the viability of RAW 264.7 cells, which was measured by MTT assay; B, NO production in RAW 264.7 cells, which was measured by Griess method; C, the representative flow cytometric profiles; D, effect of cordysinan on the pinocytosis of RAW 264.7 cells by flow cytometry. Culture medium and LPS (0.4 μg/mL) were used as a blank control and positive control, respectively. All values were expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control group.

3.4. Immunomodulatory activity of cordysinan from C. sinensis

Macrophages play indispensable roles in the innate and adaptive immune response to pathogens and in-tissue homeostasis (Hao et al., 2012). RAW 264.7 cells are a macrophage-like cell line, the experiments performed with this cell line may be a good indicator of the immunobiological activity of cordysinan. Therefore, the immunomodulatory activity of cordysinan from *C. sinensis* was evaluted on the RAW 264.7 cells. Fig. 4A showed that cordysinan had no cytotoxic effects even if at the concentration of 100 μg/mL. Therefore, the measurement of pinocytosis, NO and cytokines production of RAW 264.7 cells treated by cordysinan under the concentration could well represent the cell function. Moreover, as shown in Fig. 4B, cordysinan could promote NO production released from RAW 264.7 cells ($p < 0.05$), and induced similar contents of NO with those of LPS (0.4 μg/mL) at the concentration of 100 μg/mL, as well as the LPS-induced NO production was 51.3 ± 7.0 μM. Moreover, the effects of cordysinan on pinocytic activity of macrophages were evaluated by the flow cytometry analysis (Fig. 4C). The results demonstrated that cordysinan obviously increased pinocytic activity of RAW 264.7 cells at almost 4.6-times to the blank control (Fig. 4D). Additionally, the activated macrophages can secrete secondary compounds such as the cytokines and chemokines which allow immune cells to communicate with one another or induce infiltration of leukocytes to the site of infection (Martinez, Sica, Mantovani, & Locati, 2008). As shown in Table 2, cordysinan can promote multiple cytokines (IL-1α, IL-6, IL-10 and TNF-α) and chemokines (MCP-1, MIP-1α, IP-10 and KC) released from RAW 264.7 cells. All of these results suggested that cordysinan possessed obvious immunomodulatory activity on macrophages. Previous

Table 2
Effects of cordysinan on the cytokines/chemokines secretion from RAW 264.7 cells[a].

Cytokines/chemokines (pg/mL)	Control		LPS (μg/mL)		Cordysinan (μg/mL)	
	0	0.4	0.1	1	10	100
IL-1α	21.1 ± 4.7	139.3 ± 9.8*	157.1 ± 22.7*	252.1 ± 28.3*	257.9 ± 40.2*	296.2 ± 26.3**
IL-6	40 ± 4.8	7490.2 ± 690.8*	2441.7 ± 17.4**	2933 ± 780.7*	4762.6 ± 614*	12,548.5 ± 1323.7*
IL-10	37.5 ± 2.2	10,410.9 ± 87.1**	148.7 ± 41.5*	160.7 ± 25.8*	178.6 ± 22.5*	482.3 ± 20.1**
TNF-α	116.6 ± 27.3	11,405.6 ± 1600.3*	2405.1 ± 836*	2787.7 ± 194.8**	5448.7 ± 600.8**	9384.9 ± 1180.3*
MCP-1	1012.3 ± 71.2	3391.8 ± 447.9**	1103.6 ± 158.7	1897.6 ± 328.4*	2336.3 ± 213.2**	3923.9 ± 241.9**
IP-10	531.6 ± 40.1	1350.1 ± 220.6**	1672.3 ± 301*	3130.1 ± 297.5**	3041.5 ± 451.3*	4949.4 ± 395.8**
MIP-1α	1878.8 ± 206.9	13,713.5 ± 964.3**	8239.0 ± 1005.7**	11,108.6 ± 655.9**	12,116 ± 488.4**	17,732 ± 519**
KC	109.8 ± 29.2	466.1 ± 82.3**	112.9 ± 15.4	147.7 ± 20	202.3 ± 16.9*	222.1 ± 18.8*

[a] RAW 264.7 cells were incubated overnight, then treated with cordysinan (0.1–100 μg/mL, respectively) for 24 h, the contents of cytokines/chemokines in the culture medium were measured by Multiplex bead-based cytokine assay. Culture medium and LPS (0.4 μg/mL) were used as a blank control and positive control, respectively. Values are the mean ± SEM of three independent experiments.

* $p < 0.05$.

** $p < 0.001$ versus control.

studies suggested that the branched structures of polysaccharides were beneficial for their bioactivities (Chen et al., 2011; Yan et al., 2011), as well as the branched spherical shape (Han et al., 2011). Furthermore, the cordysinan possesses the physical properties such as the low viscosity, the high solubility and the low molecular weight, which were beneficial for it to pass through the organizational barriers to enter the interior of the cell or attach to the receptors (Han et al., 2011), and the periphery of hyperbranched cordysinan has many endgroups that can be functioned and used as sites to interact with the cells (Tao et al., 2007; Yang et al., 2010). Therefore, the obvious immunomodulatory activity of cordysinan might attribute to its hyperbranched structures and globular conformation.

4. Conclusion

A hyperbranched polysaccharide, cordysinan, was isolated from natural *C. sinensis*. The molecular parameters and the solution properties of cordysinan were determined by using SEC-MALLS and SEC-TDA. By applying the theory of polymer solutions, the results suggested that cordysinan existed as globular shape in aqueous solution, which for the first time provided fundamental information on the chain conformation of polysaccharides from natural *C. sinensis*. Moreover, cordysinan could obviously stimulate macrophages function, which may attribute to its hyperbranched structures. The structure-bioactivity relationship of polysaccharides from natural *C. sinensis* will be investigated based on the fundamental information in future.

Acknowledgments

This research was supported by grants from the Science and Technology Development Fund of Macao (FDCT059/2011/A3), and the University of Macau (MYRG140 and MYRG2014-00041) to S.P. Li.

References

Burchard, W. (1999). Solution properties of branched macromolecules. In J. Roovers (Ed.), *Branched polymers II* (Vol. 143) (pp. 113–194). Berlin, Heidelberg: Springer.

Chen, X., Cao, D. X., Zhou, L., Jin, H. Y., Dong, Q., Yao, J., et al. (2011). Structure of a polysaccharide from *Gastrodia elata* Bl., and oligosaccharides prepared thereof with anti-pancreatic cancer cell growth activities. *Carbohydrate Polymers*, *86*(3), 1300–1305.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Guan, J., Yang, F. Q., & Li, S. P. (2010). Evaluation of carbohydrates in ntural and cultured *Cordyceps* by pressurized liquid extraction and gas chromatography coupled with mass spectrometry. *Molecules*, *15*(6), 4227–4241.

Guan, J., Zhao, J., Feng, K., Hu, D. J., & Li, S. P. (2011). Comparison and characterization of polysaccharides from natural and cultured *Cordyceps* using saccharide mapping. *Analytical and Bioanalytical Chemistry*, *399*(10), 3465–3474.

Hakomori, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *Journal of Biochemistry*, *55*, 205–208.

Han, Q., Yu, Q. Y., Shi, J., Xiong, C. Y., Ling, Z. J., & He, P. M. (2011). Molecular characterization and hypoglycemic activity of a novel water-soluble polysaccharide from tea (*Camellia sinensis*) flower. *Carbohydrate Polymers*, *86*(2), 797–805.

Hao, N.-B., Lü, M.-H., Fan, Y.-H., Cao, Y.-L., Zhang, Z.-R., & Yang, S.-M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clinical and Developmental Immunology*, *2012*, 11.

Hu, D. J., Cheong, K. L., Zhao, J., & Li, S. P. (2013). Chromatography in characterization of polysaccharides from medicinal plants and fungi. *Journal of Separation Science*, *36*(1), 1–19.

Huang, Z. P., Huang, Y. A., Li, X. B., & Zhang, L. N. (2009). Molecular mass and chain conformations of *Rhizoma Panacis Japonici* polysaccharides. *Carbohydrate Polymers*, *78*(3), 596–601.

Ji, N.-F., Yao, L.-S., Li, Y., He, W., Yi, K.-S., & Huang, M. (2011). Polysaccharide of *Cordyceps sinensis* enhances cisplatin cytotoxicity in non-small cell lung cancer H157 cell line. *Integrative Cancer Therapies*, *10*(4), 359–367.

Kogan, G., Alföldi, J., & Masler, L. (1988). 13C-NMR spectroscopic investigation of two yeast cell wall β-D-glucans. *Biopolymers*, *27*(7), 1055–1063.

La Gatta, A., De Rosa, M., Marzaioli, I., Busico, T., & Schiraldi, C. (2010). A complete hyaluronan hydrodynamic characterization using a size exclusion chromatography-triple detector array system during in vitro enzymatic degradation. *Analytical Biochemistry*, *404*(1), 21–29.

Li, S. P., Li, P., Dong, T. T. X., & Tsim, K. W. K. (2001). Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured *Cordyceps mycelia*. *Phytomedicine*, *8*(3), 207–212.

Li, S.-P., Wu, D.-T., Lv, G.-P., & Zhao, J. (2013). Carbohydrates analysis in herbal glycomics. *TrAC: Trends in Analytical Chemistry*, *52*(0), 155–169.

Li, S. P., Zhang, G. H., Zeng, Q., Huang, Z. G., Wang, Y. T., Dong, T. T. X., et al. (2006). Hypoglycemic activity of polysaccharide, with antioxidation, isolated from cultured *Cordyceps mycelia*. *Phytomedicine*, *13*(6), 428–433.

Ma, Z. C., Wang, J. G., & Zhang, L. N. (2008). Structure and chain conformation of β-glucan isolated from *Auricularia auricula-judae*. *Biopolymers*, *89*(7), 614–622.

Martinez, F. O., Sica, A., Mantovani, A., & Locati, M. (2008). Macrophage activation and polarization. *Frontiers in Bioscience*, *13*, 453–461.

Ng, T. B., & Wang, H. X. (2005). Pharmacological actions of *Cordyceps*, a prized folk medicine. *Journal of Pharmacy and Pharmacology*, *57*(12), 1509–1519.

Nie, S. P., Cui, S. W., Phillips, A. O., Xie, M. Y., Phillips, G. O., Al-Assaf, S., et al. (2011). Elucidation of the structure of a bioactive hydrophilic polysaccharide from *Cordyceps sinensis* by methylation analysis and NMR spectroscopy. *Carbohydrate Polymers*, *84*(3), 894–899.

Nie, S., Cui, S. W., Xie, M., Phillips, A. O., & Phillips, G. O. (2013). Bioactive polysaccharides from *Cordyceps sinensis*: Isolation, structure features and bioactivities. *Bioactive Carbohydrates and Dietary Fibre*, *1*(1), 38–52.

Piazza, L., Bertini, S., & Milany, J. (2010). Extraction and structural characterization of the polysaccharide fraction of *Launaea acanthodes* gum. *Carbohydrate Polymers*, *79*(2), 449–454.

Scherrenberg, R., Coussens, B., van Vliet, P., Edouard, G., Brackman, J., de Brabander, E., et al. (1998). The molecular characteristics of poly(propyleneimine) dendrimers as studied with small-angle neutron scattering, viscosimetry, and molecular dynamics. *Macromolecules*, *31*(2), 456–461.

Singh, V., Kumar, P., & Sanghi, R. (2012). Use of microwave irradiation in the grafting modification of the polysaccharides – A review. *Progress in Polymer Science*, *37*(2), 340–364.

Singh, V., & Tiwari, A. (2008). Microwave-accelerated methylation of starch. *Carbohydrate Research*, *343*(1), 151–154.

Solomon, O. F., & Ciută, I. Z. (1962). Determination of the intrinsic viscosity of polymer solution by a simple determination of viscosity. *Journal of Applied Polymer Science*, *6*(24), 683–686.

Tao, Y. Z., Zhang, L., Yan, F., & Wu, X. J. (2007). Chain conformation of water-insoluble hyperbranched polysaccharide from fungus. *Biomacromolecules*, *8*(7), 2321–2328.

Tao, Y. Z., Zhang, Y. Y., & Zhang, L. N. (2009). Chemical modification and antitumor activities of two polysaccharide-protein complexes from *Pleurotus tuber-regium*. *International Journal of Biological Macromolecules*, *45*(2), 109–115.

Wang, J. G., Ma, Z. C., Zhang, L. N., Fang, Y. P., Jiang, F. T., & Phillips, G. O. (2011). Structure and chain conformation of water-soluble heteropolysaccharides from *Ganoderma lucidum*. *Carbohydrate Polymers*, *86*(2), 844–851.

Wang, Z. M., Peng, X. A., Lee, K. L. D., Tang, J. C. O., Cheung, P. C. K., & Wu, J. Y. (2011). Structural characterisation and immunomodulatory property of an acidic polysaccharide from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chemistry*, *125*(2), 637–643.

Wu, Y. L., Sun, C. R., & Pan, Y. J. (2005). Structural analysis of a neutral (1–>3),(1–>4)-beta-D-glucan from the mycelia of *Cordyceps sinensis*. *Journal of Natural Products*, *68*(5), 812–814.

Wu, Y. L., Sun, C. R., & Pan, Y. J. (2006). Studies on isolation and structural features of a polysaccharide from the mycelium of an Chinese edible fungus (*Cordyceps sinensis*). *Carbohydrate Polymers*, *63*(2), 251–256.

Wu, D.-T., Xie, J., Hu, D.-J., Zhao, J., & Li, S.-P. (2013). Characterization of polysaccharides from *Ganoderma* spp. using saccharide mapping. *Carbohydrate Polymers*, *97*(2), 398–405.

Xu, X. J., Chen, P., Wang, Y., & Zhang, L. (2009). Chain conformation and rheological behavior of an extracellular heteropolysaccharide Erwinia gum in aqueous solution. *Carbohydrate Research*, *344*(1), 113–119.

Xu, X. J., Chen, P., Zhang, L. N., & Ashida, H. (2012). Chain structures of glucans from *Lentinus edodes* and their effects on NO production from RAW 264.7 macrophages. *Carbohydrate Polymers*, *87*(2), 1855–1862.

Yan, J. K., Wang, W. Q., Li, L., & Wu, J. Y. (2011). Physiochemical properties and antitumor activities of two α-glucans isolated from hot water and alkaline extracts of *Cordyceps* (Cs-HK1) fungal mycelia. *Carbohydrate Polymers*, *85*(4), 753–758.

Yan, J. K., Wang, W. Q., Ma, H. L., & Wu, J. Y. (2013). Sulfation and enhanced antioxidant capacity of an exopolysaccharide produced by the medicinal fungus *Cordyceps sinensis*. *Molecules*, *18*(1), 167–177.

Yang, L. Q., Fu, S. S., Zhu, X. N., Zhang, L. M., Yang, Y. R., Yang, X. M., et al. (2010). Hyperbranched acidic polysaccharide from green tea. *Biomacromolecules*, *11*(12), 3395–3405.

Yang, L. Q., & Zhang, L. M. (2009). Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. *Carbohydrate Polymers*, *76*(3), 349–361.

Yu, H. M., Wang, B. S., Huang, S. C., & Duh, P. D. (2006). Comparison of protective effects between cultured *Cordyceps militaris* and natural *Cordyceps sinensis* against oxidative damage. *Journal of Agricultural and Food Chemistry*, *54*(8), 3132–3138.

Zhang, Y., Li, S., Wang, X., Zhang, L., & Cheung, P. C. K. (2011). Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocolloids*, *25*(2), 196–206.

Zhong, S., Pan, H. J., Fan, L. F., Lv, G. Y., Wu, Y. Z., Parmeswaran, B., et al. (2009). Advances in research of polysaccharides in *Cordyceps* species. *Food Technology and Biotechnology*, *47*(3), 304–312.

Zhou, X. W., Gong, Z. H., Su, Y., Lin, J., & Tang, K. X. (2009). *Cordyceps* fungi: Natural products, pharmacological functions and developmental products. *Journal of Pharmacy and Pharmacology*, *61*(3), 279–291.

Zhu, J. S., Halpern, G. M., & Jones, K. (1998a). The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*-Part II. *Journal of Alternative and Complementary Medicine*, *4*(4), 429–457.

Zhu, J. S., Halpern, G. M., & Jones, K. (1998b). The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis* Part I. *Journal of Alternative and Complementary Medicine*, *4*(3), 289–303.