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Purification, characterization and immunostimulatory activity of polysaccharide from *Cipangopaludina chinensis*

Qingping Xiong[a], Yunpeng Jiao[b], Xirong Zhao[a], Xiaoming Chen[a], Qianghua Zhang[a], Changxing Jiang[a,*]

[a] College of Life Science and Chemical Engineering, Huaiyin Institute of Technology, Huaian, Jiangsu 223003, PR China
[b] Food Engineering Department, Jiangsu Food Science College, Huaian, Jiangsu 223003, PR China

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ABSTRACT

In this study, we investigated the purification, preliminary characterization and immunostimulatory activity *in vivo* of polysaccharide from *Cipangopaludina chinensis* (CCPS). Firstly, crude CCPS was prepared by hot water extraction. And the crude CCPS was sequentially purified by chromatography of DEAE-52 and Sephadex G-100, resulting in two purified fractions of CCPS-1 and CCPS-2. We found the two fractions were homogeneous heteropolysaccharides mainly composed of rhamnose and glucose with the average molecular weight of 226 and 235 kDa, respectively. CCPS-2 was quite different from CCPS-1. It had much higher content of uronic acid and sulfuric radical. For immunostimulatory activity *in vivo*, crude CCPS could significantly increase the thymus and spleen indices, enhance the macrophage function, and increase the level of serum hemolysin in cyclophosphamide-treated mice, suggesting CCPS had a potent immunostimulatory activity and could be explored as a potential natural immunomodulatory agent

1. Introduction

Cancer is one of the leading causes of death in the world (Wang et al., 2011). Chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer. However, they can harm healthy cells and cause side effects such as myelosuppression and immunosuppression, which limit their use in tumor treatment (Chen et al., 2012). Recently, polysaccharides isolated from natural source have been shown to profoundly affect the immune system with relative nontoxicity and no significant side effects, and therefore are ideal candidates as immunomodulator with a wide application (Chen, Yang, et al., 2010; Chen, Tang, Chen, Wang, & Li, 2010; Yang, Zhao, & Lv, 2008).

Cipangopaludina chinensis, one of invertebrates, is a common group of vivipard gastropod and widespread in China and many other countries. This species resides in pools, lakes, streams and other water bodies and lives on organic particles and microbes (Chiu, Chen, Lee, & Chen, 2002; Li, 2012; Sastre et al., 2007). Its fresh meat contains high amounts of protein, taurine, essential amino acids, calcium, iron and zinc and is traditionally used as food supplement in China (Cao & Yao, 2005; Mulia et al., 2009). It has been processed into many kinds of foods by canning, saucing or cooking. In recent years, it has been used in the preparation of foods such as meat sauce and jerky to improve its commercial value (Liu, 2002; Zhang, Wu, & Wang, 2005). In addition, *C. chinensis* have a long history as a traditional medicine for the treatment of diabetes and liver diseases in China (Zhao, 2011). Recent studies have demonstrated that extracts of *C. chinensis* meat exert various pharmacological effects, such as anticancer and immunostimulatory activities (Fu, Jiang, & Zhang, 2010). However, little attention has been devoted to the characterization and immunostimulatory activity of polysaccharides from *C. chinensis* (CCPS). Therefore, we report here the purification, characterization and immunostimulatory activity *in vivo* of CCPS. Firstly, crude CCPS was purified by ion-exchange chromatography and size-exclusion chromatography, and the purified factions were then characterized by chemical analysis, gas chromatography (GC), fourier transform-infrared spectroscopy (FT-IR) and high performance liquid chromatography (HPLC). Finally, the immunostimulatory activity *in vivo* of crude CCPS was investigated by using the cyclophosphamide-treated mice model.

2. Materials and methods

2.1. Materials and reagents

C. chinensis was purchased from Huaian Aquatic Product Market (Huaian, China). Arabinose, fucose, galactose, glucose, glucuronic acid, mannose, rhamnose, xylose, DEAE-52 cellulose and Sephadex G-100 were purchased from Sigma Chemical Co. (St. Louis, MO,

* Corresponding author. Tel.: +86 517 83559216; fax: +86 517 83559216.
E-mail address: j1c2x3@gmail.com (C. Jiang).

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USA). Cyclophosphamide (CPA) was obtained from Jiangsu Hengrui Medcine Co. (Nanjing, China). Dialysis tube (molecular weight cut-off, 12,000–14,000 Da) was purchased from Shanghai Green Bird Science and Technology Development Co., Ltd. (Shanghai, China). All other reagents were of analytical grade.

2.2. Animals

The male Kunming mice were purchased from the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). They were housed in an air-conditioned animal room with constant temperature (21 ± 1 °C and 50–60% relative humidity in a 12 h light/dark cycles, and fed laboratory chows and water *ad libitum*. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (The Ministry of Science and Technology of China, 2006). All efforts were made to minimize animal's suffering and to reduce the number of animals used.

2.3. Preparation of crude CCPS

The crude CCPS was prepared according to the reported method with some modifications (Jiang, Wang, Liu, Gan, & Zeng, 2011). Briefly, fresh *C. chinensis* was collected and washed carefully with cold water. After removing the shells and impurities, the flesh was crushed by a high speed disintegrator and the homogenate was kept in 90% of ethanol (v/v) for two weeks. Then, the collected flesh was air-dried at 50 °C and extracted with distilled water in a ratio of 1:25 (raw material to water, w/v) for 3 h at 90 °C for three times. After treatment, the mixture was centrifuged at 5000 rpm for 20 min, and the insoluble residue was treated again as mentioned above. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator, deproteinated by the method of Sevag (Sevag, Lackman, & Smolens, 1938) and mixed with three times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording the crude CCPS.

2.4. Purification of crude CCPS

The crude CCPS was sequentially purified by chromatography of DEAE-52 and Sephadex G-100 according to the reported methods with slight modifications (Qiao et al., 2009). Briefly, the solution of crude CCPS (3 ml, 15 mg/ml) was applied to a column (2.6 cm × 30 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0, 0.2 and 0.4 M sodium chloride solutions at a flow rate of 60 ml/h. The obtained elute (10 ml/tube) was collected automatically and the carbohydrates were detected by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). As results, two fractions of polysaccharides (Fig. 1) were obtained. Each fraction was collected, concentrated, dialyzed and further purified through a column (2.6 cm × 60 cm) of Sephadex G-100, respectively, resulting in two purified fractions (CCPS-1 and CCPS-2). Finally, CCPS-1 and CCPS-2 were lyophilized for further study.

2.5. Preliminary characterization of CCPS

2.5.1. Determination of contents of total sugars, sulfate, protein and uronic acid

The total sugar content of CCPS was determined using the phenol-sulphuric acid method (Dubois et al., 1956). The protein content was determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The content

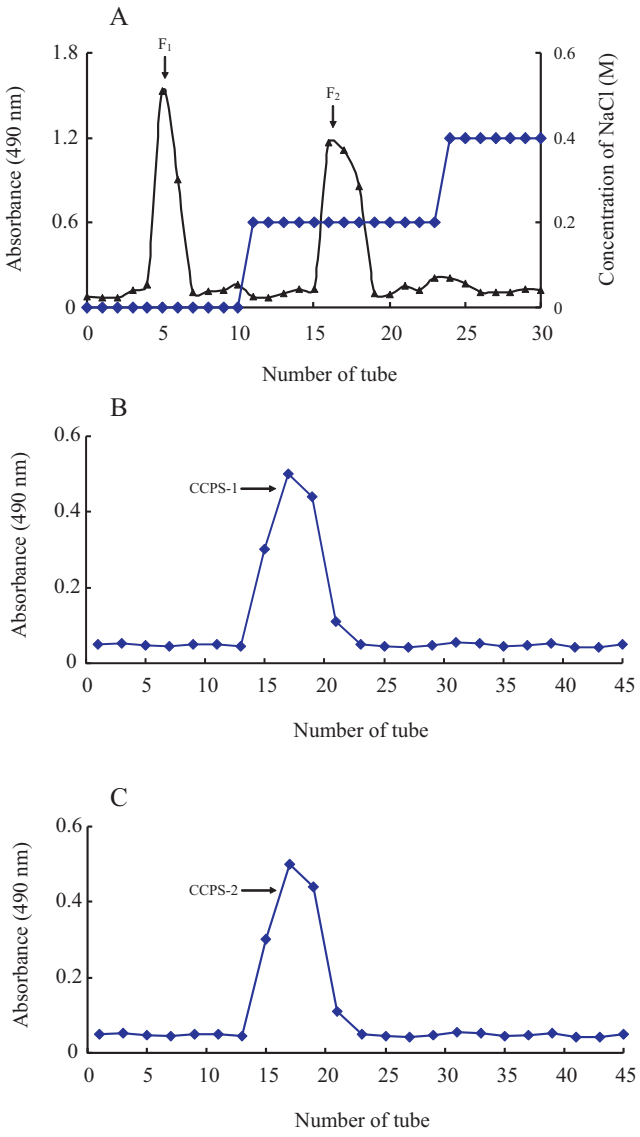

Fig. 1. Stepwise elution curve of crude CCPS on anion-exchange chromatography column of DEAE-52 cellulose (A) and elution curve of polysaccharide fractions (F_1 and F_2) from DEAE-52 cellulose on size-exclusion chromatography column of Sephadex G-100 (B and C).

of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (Blumenkrantz & Asboe-Hansen, 1973) using D-glucuronic acid as the standard. The content of sulfate radical was determined according to the reported method (Doigson & Price, 1962).

2.5.2. Determination of molecular weight

The molecular weight determination of CCPS was performed using a size-exclusion HPLC chromatography instrument (Agilent 1100, USA) equipped with a refractive index detector (RID). All samples (10.0 mg) were dissolved in distilled water (1.0 ml), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of TSK-GEL G3000SW$_{xl}$ (7.5 mm × 300 mm, Tosoh Corp., Japan). The column was maintained at a temperature of 25 °C and eluted with 0.1 M Na_2SO_4 solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.8 ml/min. For preparing molecular weight calibration curve, P-5, P-10, P-20, P-100, P-200, P-400 and P-800 (Showa Denko K.K., Japan) were used as the molecular weight References

2.5.3. FT-IR spectral analysis

FT-IR spectrum of CCPS was recorded with a Nicolet 6700 FT-IR Spectrometer (Thermo Co., USA) using KBr disks method (Wang et al., 2004). Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with potassium bromide (KBr) powder and then pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.5.4. Analysis of monosaccharide composition of CCPS

The monosaccharide composition of CCPS was determined using the method reported by Gan, Ma, Jiang, Xu, and Zeng (2011) with slight modification. Briefly, the polysaccharide sample (5.0 mg) was hydrolyzed with 4 ml trifluoroacetic acid (TFA, 2 M) at 120 °C in an oven for 2 h, and the excess TFA was removed by evaporation at a temperature of 40 °C. Then, the hydrolyzate was repeatedly co-concentrated with methanol to dryness and acetylated by the addition of a mixture of methanol, pyridine and acetic anhydride. The monosaccharide standards including rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose were acetylated in the same way. Finally, the acetylated samples were analyzed by a 7890 N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The oven temperature was maintained at 120 °C for 3 min, and then increased gradually to 210 °C at a rate of 3 °C/min. The temperatures of detector and injector were set at 280 °C and 250 °C, respectively. The flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively.

2.6. Evaluation of immunostimulatory effect of crude CCPS

2.6.1. Assay of immune organ index and carbon clearance

The assay of immune organ index and carbon clearance of crude CCPS were carried out according to the reported methods with some modifications (Kuang, Xia, Yang, Wang, & Wang, 2011). Briefly, the mice were randomly divided into six groups (10 for each). Group I was normal control given gastric gavage of 0.9% NaCl (25 ml/kg BW) once daily for 14 days and hypodermic injection of 0.9% NaCl (15 ml/kg BW) on days 6, 7 and 8. Group II were administrated with 0.9% NaCl (25 ml/kg BW) once daily for 14 days and with CPA by hypodermic injection (40 mg/kg BW) on days 6, 7 and 8. Group III was treated with 80 mg/kg BW of *Poria cocos* polysaccharides. Group IV, V and VI were administrated with crude CCPS at dose of 200, 400 and 600 mg/kg BW by gastric gavage (25 ml/kg BW) respectively once daily and with CPA by hypodermic injection (40 mg/kg BW) on days 6, 7 and 8. After pretreatment with drugs for 14 days, the fine carbon suspension prepared by dilution of black India ink (1 ml) with saline (2 ml) was injected at a dose of 10 ml/kg BW into the tail vein of each mouse. 20 μl of blood was drawn from postobital vein at two time points (0.5 and 6 min). The blood samples were moved to the tubes filled with 2 ml of 0.1% Na_2CO_3 and the absorbance was measured at 675 nm. All the mice were weighed and killed by decapitation. The spleen and thymus were excised and weighed immediately. The thymus and spleen indices and phagocytosis index were calculated according to the following formula:

$$\text{Thymus and spleen indices} = 100 \times \frac{W_1}{W_0}$$

$$\text{Phagocytosis index} = \left(\frac{\log A_1 - \log A_2}{t_2 - t_1}\right)^{1/3} \times \frac{W_0}{W_{XL}}$$

where W_1 is the weight of spleen or thymus, W_0 is the weight of body, W_{XL} is the total weight of liver and spleen of each mouse, A_1 and A_2 is the absorbance at 0.5 min (t_1) and 6 min (t_2), respectively.

2.6.2. Serum hemolysin analysis

The serum hemolysin was analyzed by the method described by Wen, Zhang, Liu, Guo and Wang (2006). Briefly, mice were grouped and pretreated with drugs as described in Section 2.6.1. On the 8th day of administration, the mice except for the control group were sensitized by injection of 0.2 ml SRBC (10%, v/v). After 6 days, mice were weighed and killed by decapitation. The blood samples were collected immediately and centrifuged at 3000 rpm at 4 °C for 10 min to afford the serums. 20 μl of serum was diluted to 500 times with normal saline. 1 ml of the diluted serum samples was mixed with 0.5 ml of 10% SRBC and 1 ml of fresh guinea pig serum (1:10 dilution) in the reaction tubes. After incubation for 1 h at 37 °C, the reaction tubes were moved into ice bath immediately and centrifuged at 3000 rpm at 4 °C for 10 min. 1 ml of the supernatant was mixed with 3 ml of Drabkin's solution (1.0 g $NaHCO_3$, 0.05 g KCN, 0.2 g $K_3Fe(CN)_6$ mixed in 1 L distilled water). Blank control (without mice serum) and half hemolysis control (0.25 ml SRBC) were also synchronously prepared. The final mixture was kept at room temperature for 10 min and absorbance at 540 nm was read against the blank control. The level of serum antibody was calculated according to the following formula:

$$\text{Serum antibody level} = 500 \times \frac{A_1}{A_2}$$

where A_1 and A_2 is the absorbance at 540 nm of sample and half hemolysis, respectively.

2.7. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests. *P*-Values of less than 0.05 were regarded as significant. All statistical analyses were carried out by using SPSS for Windows, Version 13.0 (SPSS, Chicago, IL).

3. Results and discussion

3.1. Preparation of polysaccharide fractions

The CCPS was prepared from *C. chinensis* by hot water extraction, ethanol precipitation and vacuum freeze-drying. The overall yield of CCPS was 8.7% based on the dried fresh used. The crude CCPS was firstly separated through a DEAE-52 anion-exchange column. As a result, two independent elution peaks (F_1 and F_2) as shown in Fig. 1A were obtained. The two fractions were collected, concentrated, dialyzed and loaded onto a Sephadex G-100 column, respectively. The column was eluted with deioned water, and the resulting elute was collected. As shown in Fig. 1B and C, each fraction generated one single elution peak, affording CCPS-1 and CCPS-2, respectively.

3.2. Molecular weight of polysaccharide fractions

For determination of the homogeneity and molecular weight of CCPS-1 and CCPS-2, an Agilent 1100 HPLC system was used. As shown in Fig. 2, the HPLC profile of each purified fraction had a single and symmetrical narrow peak, which indicated that they were homogeneous polysaccharides. In addition, the average molecular weights of CCPS-1 and CCPS-2 were estimated to be 226 and 235 kDa, respectively.

3.3. FT-IR spectroscopy of polysaccharide fractions

An FT-IR spectroscopy is used to investigate the vibrations of molecules and polar bonds between the different atoms. It is possible to analyze the structures of polysaccharides such as

Table 1
The chemical compositions of CCPS-1 and CCPS-2.

Sample	Molecular eight (kDa)	Carbohydrate (%)	Protein (%)	Uronic acid (%)	Sulfuric radical (%)	Monosaccharide composition (%)		
						Rhamnose	Xylose	Glucose
CCPS-1	226	98.2	1.4	–[a]	1.5	1.2	0.7	98.1
CCPS-2	235	85.5	2.7	3.2	2.6	38.4	–	61.6

[a] Not detected.

monosaccharide types, glucosidic bonds and functional groups using an FT-IR spectroscopy (Yang & Zhang, 2009). CCPS-1 and CCPS-2 were characterized by FT-IR spectroscopy (Fig. 3). A strong and broad absorption peak at 3427 cm^{-1} for O—H stretching vibrations, a peak at 2928 cm^{-1} for C—H stretching vibrations, and a strong extensive absorption in the region of 900–1200 cm^{-1} for coupled C—O and C—C stretching and C—OH bending vibrations were observed in CCPS-1 and CCPS-2, indicating the characteristic absorptions of polysaccharides (Liu et al., 2008). The absorption peak at 1240 cm^{-1} and 1155 cm^{-1} was assigned to the asymmetric stretching vibrations of SO, an evidence of sulfate ester, confirming directly that CCPS-1 and CCPS-2 were sulfated polysaccharides (Qiao et al., 2009). The bands at 1635 cm^{-1} and 1339 cm^{-1} were characteristic signals for the deprotonated carboxylic group (COO^-), indicating CCPS-2 being acidic polysaccharide (Gan, Ma, Jiang, Xu, & Zeng, 2011). Finally, there was an absorption peak at about 850 cm^{-1} in the FT-IR spectrum of CCPS-1 indicating the presence of α-type glycosidic linkages in CCPS-1 (Li et al., 2008). The glycosidic linkage type of CCPS-1 was similar to that of polysaccharide from *Hyriopsis cumingii* (Qiao et al., 2009).

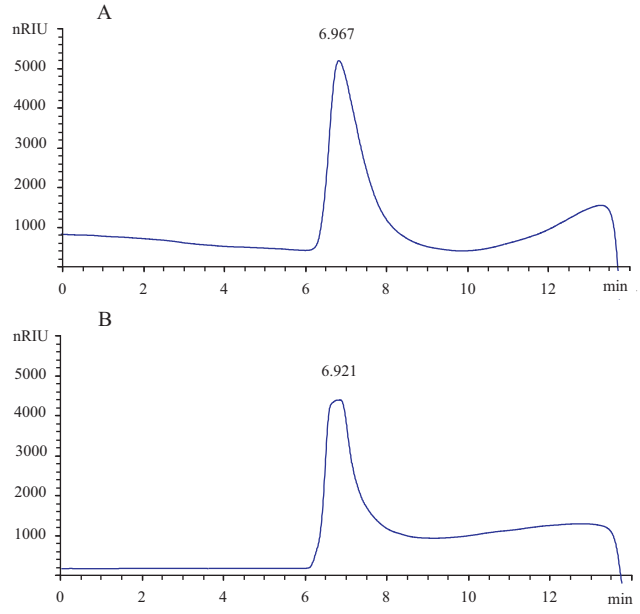

Fig. 2. HPLC profiles of CCPS-1 (A) and CCPS-2 (B).

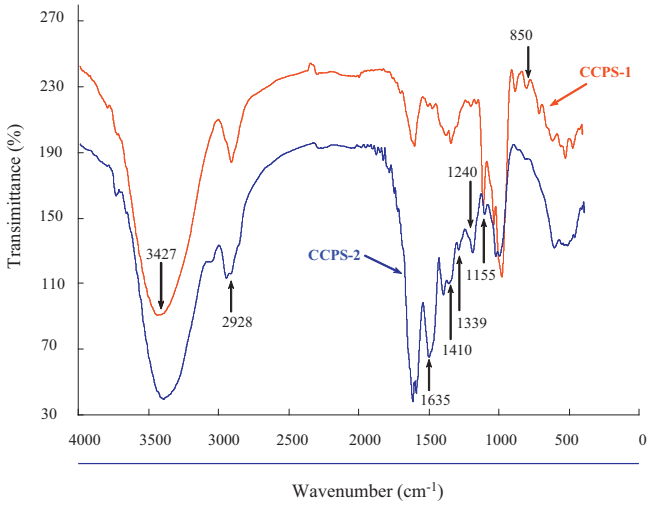

Fig. 3. FT-IR spectra of CCPS-1 (A) and CCPS-2 (B).

3.4. Chemical compositions of polysaccharide fractions

The results of chemical analysis showed that the content of total sugar for CCPS-1 and CCPS-2 were 98.2% and 85.5%, respectively. The content of protein for CCPS-1 and CCPS-2 were 1.4% and 2.7%, respectively. CCPS-2 had relatively high amount of sulfate than CCPS-1. The content of uronic acid for CCPS-2 was 3.2%. However, no uronic acid was detected in CCPS-1 (Table 1). The results were in accordance with those obtained from FT-IR analysis.

The monosaccharide composition of CCPS-1 and CCPS-2 was determined by GC, and the monosaccharides in the hydrolytes were identified by comparing the retention times with those of standards (Fig. 4A). As shown in Fig. 4B, CCPS-1 was composed of rhamnose, xylose and glucose in a molar percent of 1.2%, 0.7%, and 98.1%, respectively. CCPS-2 was composed of rhamnose and glucose in a molar percent of 38.4% and 61.6%, respectively (Fig. 4C). Other sugars, such as arabinose, fucose, mannose and galactose were not found in CCPS-1 and CCPS-2.

The acidity of polysaccharides eluted from an anion-exchange column is associated with salt concentration of elution solution (Yang, He, et al., 2008). Since CCPS-1 and CCPS-2 were eluted from a DEAE-52 anion-exchange column with elution solution at increased NaCl concentrations (0 and 0.2 M, respectively), CCPS-1 was regarded as a neutral polysaccharide, while CCPS-2 was acidic polysaccharides. The results were in coincidence with the results from the uronic acid analysis for polysaccharide fractions: CCPS-1 contained a small amount of uronic acid, while CCPS-2 contained a relatively large amount of uronic acid. The results indicated that the acidity of these polysaccharides was probably due to the presence of uronic acid. The types of uronic acid residues in CCPS-2 should be investigated in detail in future works.

3.5. Immunostimulatory activity of crude CCPS in vivo

Polysaccharides are a structurally diverse class of macromolecules, and this structural variability can profoundly affect their cell-type specificity and their biological activity in T lymphocytes, B lymphocytes and macrophages (Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005). It is well known that CPA is an important chemotherapeutic drug in tumor treatment, but it is adverse to immune system of organism and leads to immunosuppression, which sometimes are life-threatening (Wang et al., 2011). Poria cocos, a fungus belonging to the *Polyporaceae* family, is one of the most important herbs in China and other Asian countries. It has been reported that polysaccharides extracted from

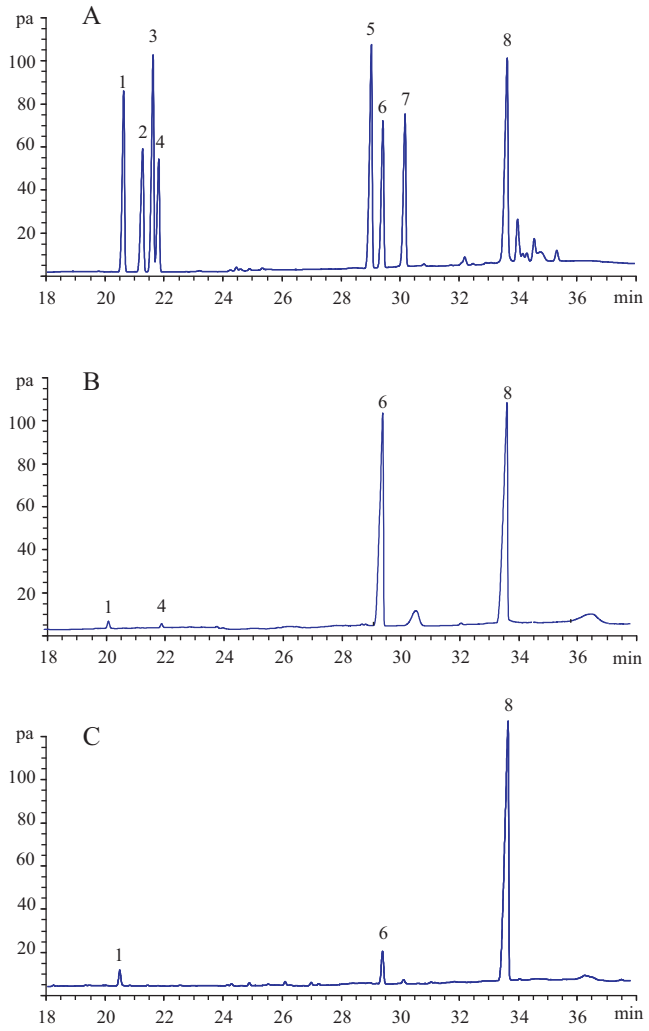

Fig. 4. GC chromatograms of monosaccharide reference (A), CCPS-1 (B) and CCPS-2 (C). 1, Rhamnose; 2. Arabinose; 3, Fucose; 4, Xylose; 5, Mannose; 6, Glucose; 7, Galactose; 8, Inositol.

Poria cocos (mainly glucan) have antioxidant and immunological activities (Chen, Tang, et al., 2010; Jin et al., 2003). In this study, therefore, mice treated with CPA were used as immunosuppression animal model and *Poria cocos* polysaccharides commercially available were used as positive control medicine.

Immune system degeneration is expressed as an aging process from center to periphery piloted by thymus involution. So, thymus decline is an important marker of immune system aging. Some immune organs including spleen and thymus will degenerate in volume, weight and function with the aging of life. Many immunosuppressants may cause decline of immune organ. On the other hand, these degenerations of immune organ can be regained by some immunoenhancers. In the present study, the effects of crude CCPS on the thymus and spleen indices were measured. As shown in Table 2, the indices of spleen and thymus of model control group mice were significantly decreased compared with those of the normal control group mice ($P < 0.05$). On the contrary, the indices of spleen and thymus were significantly increased in the groups by pretreatment with crude CCPS (especially at a dose of 600 mg/kg BW). The results indicated that crude CCPS was able to act against the immunosuppression induced by CPA.

Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defense. Macrophages are an integral part of the immune system, acting

Table 2
The effects of crude CCPS on the thymus and spleen indices of the CPA-treated mice.

Group	Thymus and spleen indices	
	Spleen index (mg/g)	Thymus index (mg/g)
I	2.32 ± 0.37^{a}	1.97 ± 0.27^{a}
II	1.53 ± 0.47^{c}	1.21 ± 0.24^{d}
III	2.27 ± 0.89^{a}	1.62 ± 0.13^{b}
IV	1.67 ± 0.39^{bc}	1.23 ± 0.26^{d}
V	1.65 ± 0.36^{bc}	1.42 ± 0.34^{c}
VI	1.83 ± 0.53^{b}	1.61 ± 0.12^{b}

Mice were randomized into six groups with ten mice each. Group I was treated with saline and used as control and the other five groups were administrated with CPA by hypodermic injection (40 mg/kg BW) once daily on days 6, 7 and 8. Group II was used as model treated 25 ml/kg BW of normal saline. Group III was used as positive control and treated with 80 mg/kg BW of *Poria cocos* polysaccharides. The experimental groups (IV, V and VI) were administrated with CCPS at the different doses of 200, 400 and 600 mg/kg BW, respectively. After pretreatment with CCPS for 14 consecutive days, mice were weighted and sacrificed. The weights of spleen and thymus were recorded and index was expressed as relative organ weights (mg/g). Data within a column without the same superscripts (a–d) differ significantly ($P < 0.05$).

as phagocytic, microbicidal and tumoricidal effector cells. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response (De la Fuente, 2002). Macrophages also present antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (Salman et al., 2000). To observe the effect of crude CCPS on the activation of macrophages, carbon clearance tests were performed. As seen in Fig. 5A, the phagocytic index of the model group was lower compared to the control group, indicating that CPA at the dose of 40 mg/kg BW successfully induced immunosuppression in mice. Crude CCPS effectively increased the phagocytic index of CPA-treated mice dose-dependently. Especially at the high dose of crude CCPS (600 mg/kg BW), the phagocytic activity was restored to the normal level, demonstrating that crude CCPS could enhance the macrophage function in CPA-treated mice.

Humoral immunity is critical to host immunity against tumors and infection. A variety of antibodies from the blood induce effector cells to recognize and kill tumor cells and pathogens (Wang et al., 2011). Level of hemolysin in the serum reflects humoral immunity and can be used as a measure to evaluate the functional status of the humoral immune response such as antigen recognition, activation and expression (Silkworth & Loose, 1981). To determine whether administration of crude CCPS affected the regulation of hemolysin, mice were immunized with SRBC (T-dependent particulate antigen). The level of serum hemolysin in response to SRBC of the control mice and the mice administrated with crude CCPS are shown in Fig. 5B. The HC_{50} was significantly decreased in the model group compared with the control group ($P < 0.05$), indicating that the model caused by SRBC has been made successfully. The administration of crude CCPS with high dose (600 mg/kg) could significantly increase HC_{50} compared with model group ($P < 0.05$), suggesting a markedly stimulating action of crude CCPS on humoral immune response. It has been demonstrated that some botanical polysaccharides can increase humoral immune response by activation of B cells or production of specific IgA, IgM, and IgG (Yang et al., 2009). In the previous study, we found that crude CCPS could increase the serum hemolysin level, but the mechanism still needs to be studied.

The present investigation suggested that crude CCPS had immunostimulatory activity and could increase the thymus and spleen indices, enhance macrophage function, and increase the level of serum hemolysin in response to SRBC in CPA-treated

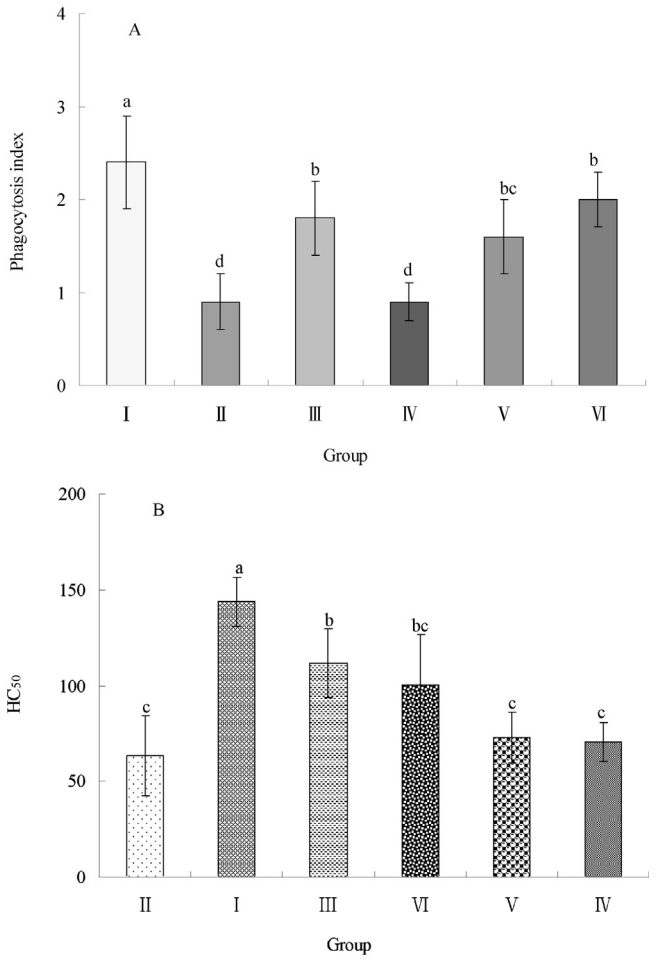

Fig. 5. Phagocytotic function of macrophages (A) and serum hemolysin formation (B) in the immunosuppressed mice stimulated with crude CCPS. Mice were randomized into six groups with ten mice each. Group I was treated with saline and used as control and the other five groups were administrated with CPA by hypodermic injection (40 mg/kg BW) once daily on days 6, 7 and 8. Group II was used as model treated 25 ml/kg BW of normal saline. Group III was used as positive control and treated with 80 mg/kg BW of *Poria cocos* polysaccharides. The experimental groups (IV, V and VI) were administrated with CCPS at the different doses of 200, 400 and 600 mg/kg BW, respectively. After pretreatment with CCPS for 14 consecutive days, mice were weighted and sacrificed, and phagocytic index of carbon particles and serum antibody level response to SRBC (HC_{50}) were determined. Values are presented as means ± SD ($n = 10$). And different alphabets (a-d) in superscript for each group denote significant difference ($P < 0.05$).

mice. It has been demonstrated that immunostimulatory activity of polysaccharides is relative to the chemical and monosaccharide composition, molecular weight and function groups of polysaccharides. Polysaccharides with high protein content are found to possess immunostimulatory activity (Qiao et al., 2010). And polysaccharides composed of glucan have been proved to stimulate the immune system (Kuang, Xia, Yang, Wang, & Wang, 2011). Furthermore, relatively high molecular weight of the polysaccharides is desired for their immunostimulatory action (Ohta et al., 2007; Pugh, Ross, ElSohly, ElSohly, & Pasco, 2001). In order to well-understand the relationship between the immunostimulatory activity and physicochemical property of polysaccharids, CCPS was isolated, purified by DEAE-52 cellulose and Sephadex G-100 and characterized by chemical analysis, HPLC, GC and FT-IR. We found that the two CCPS fractions (CCPS-1 and CCPS-2) were mainly composed of glucose with high sulfate content and relatively high molecular weight. The chemical and structural properties of CCPS might contribute to their immunostimulatory activity. It has been reported that many factors affected the activities of polysaccharides such as configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides and position of branching points (Hua, Zhang, Fu, Chen, & Chan, 2004; Huang, Jin, Zhang, Cheung, & Kennedy, 2007). Therefore it is necessary to define the complete structure of CCPS, which will certainly present a good opportunity to elucidate the biological roles of polysaccharides and develop potential immunomodulatory drugs based on the three-dimensional structures. However, the complete structure of CCPS has not been obtained and the exact underlying mechanism of CCPS is unknown. Further research on structure and structure–function relationship of CCPS is in progress.

4. Conclusion

In the present study, crude CCPS was prepared by hot water extraction and further fractionated by DEAE-cellulose column chromatography and Sephadex G-100 column chromatography into two purified fractions of CCPS-1 and CCPS-2. They were mainly composed of rhamnose and glucose with the average molecular weight of 226 and 235 kDa, respectively. Furthermore, crude CCPS have been demonstrated to have potent immunostimulatory activity *in vivo*, resulting in increasing the thymus and spleen indices, enhancing the macrophage function, and increasing the level of serum hemolysin in response to SRBC in CPA-treated mice.

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