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Characterization and antioxidant activities of polysaccharides from *Panax japonicus* C.A. Meyer

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ABSTRACT

Polysaccharides named as CP-1a and CP-2a were extracted and isolated from the rhizomes of *Panax japonicus* C.A. Meyer, a well-known Chinese traditional medicine and was used extensively by Tujia nationality. The molecular weight of CP-1a was determined by size-exclusion HPLC chromatography system, with an average molecular weight of about 6383 Da. The analysis of monosaccharide composition in the polysaccharide by HPLC chromatography revealed that CP-1a and CP-2a were a heteropolysaccharide and consisted of D-glucose and D-galactose. IR spectra indicated both CP-1a and CP-2a were the polysaccharide which contains pyranose ring. Their antioxidant activities were evaluated by various established in vitro systems, including scavenging activity of superoxide anion, hydroxyl radical and DPPH radicals. Available data obtained with in vitro models suggested that among the two samples, both showed inhibitory effects on superoxide, hydroxyl and DPPH radical. Compare CP-1a with CP-2a, the former has a strong scavenging ability.

1. Introduction

The rhizome of *Panax japonicus* C.A. Meyer, a Chinese traditional medicine, is used to cure injuries from falls by Tujia nationality, a minority nationality in China. The endangered species *P. japonicus* C.A. Meyer was found in Hubei, Sichuan, Yunnan and Guangxi provinces at lower altitudes regions, which has a bamboo-like long horizontally creeping rhizome (Morita et al., 1983). There are a few studies on the analysis of chemical components of *P. japonicus* C.A. Meyer and its allied species. The components of *P. japonicus* C.A. Meyer are polysaccharides and saponins. Nowadays, saponins obtained in *P. japonicus* C.A. Meyer were centrally studied, which have anti-ulcer and anti-obesity effects (Han, Zheng, Yoshikawa, Okuda, & Kimura, 2005; Yamahara, Kubomura, Miki, & Fujimura, 1987). Besides, it was reported that five polysaccharide samples were isolated from Rhizoma *panacis japonici* by using different methods (Huang & Zhang, 2009). Polysaccharides isolated from *P. japonicus* rhizomes have conspicuous effects on reticuloendothelial system activation (Ohtani et al., 1989).

It is known that antioxidant activity means that some antioxidants can protect cells against the damaging effects of reactive oxygen species (ROS) (Zhang et al., 2012), such as singlet oxygen, superoxide and hydroxyl radicals. The radicals also play a role in the process of ageing and carcinogenesis (Cuzzocrea, Riley, Caputi, & Selvemini, 2001). Thus, research and explore potent natural compounds with antioxidant and low cytotoxicity from plants has become an very important branch of biomedicine (Zhong et al., 2010). *P. japonicus* rhizomes are widely consumed in China; however, the chemical analysis and antioxidant activities of polysaccharides have been received little attention. In our previous reports, the polysaccharides extracted from *P. japonicus* rhizomes have been found to possess antioxidant activities (Wang et al., 2012).

The objective of this study is to isolate and purify the polysaccharides of *P. japonicus* rhizomes. Purified polysaccharides obtained were analyzed, and their associated antioxidant capabilities were evaluated.

2. Materials and methods

2.1. Materials

The *P. japonicus* were purchased from Enshi, Hubei Province, China. The following were bought from commercial sources: DEAE Cellulose (Shanghai resin plant, China), Sephadex G-75 (Amersham Pharmacia BiotechAB, Sweden); DPPH and T-series dextran were purchased from Sigma Chemical Co. (St. Louis, MO, USA); All of other reagents were analytical grade from Shanghai Chemical Reagent (P.R. China).

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2.2. Extraction, separation and purification of the polysaccharides

P. japonicus rhizomes were refluxed with 85% ethanol at 70 °C in a water bath for 3 h. After incubation, mixtures were centrifuged at 8000 rpm/min for 10 min. The insoluble residue was dried in an oven at 50 °C and extracted according to the enzyme method (Ge et al., 2009; Xu, Chen, & Chen, 2009). Protein was removed by Sevag method (Sevag, Lackman, & Smolens, 1938). D101 macroporous resin was used to remove pigments. After eluate was concentrated in vacuo to 30% of original volume, four times the volume of ethanol was added to the water and kept at 4 °C overnight in a refrigerator to precipitate polysaccharide. The precipitate was separated by centrifugation (10,000 rpm for 15 min) and air dried.

The crude polysaccharides above were redissolved in ultra-pure water, then applied to a DEAE-Cellulose anion-exchange column (2.4 cm × 24 cm) for separation with the deionized water, 0.05 M NaCl, 0.1 M NaCl, 0.2 M NaCl at a flow rate of 2 mL/min. Fractions (24 mL, each) were collected and combined according to the total carbohydrate content determined by the Phenol-Sulfuric acid method (Dubois et al., 1956). The fractions obtained were concentrated, dialyzed against distilled water with dialysis tubing (molecular weight cut-off, 3500 Da), concentrated, and then lyophilized. The major fractions were each dissolved in 0.1 M NaCl and fractionated further by gel filtration over a Sephadex G-75 (1.5 cm × 60 cm) eluting with 0.1 M NaCl at a flow rate of 1 mL/min. The major fractions were collected, freeze-dried and stored in sealed container under room temperature.

2.3. Homogeneity and molecular weight determination

Homogeneity and molecular weight was performed using a size-exclusion HPLC chromatography system (Varian 210). The following system was used: PL aquagel-OH Aqueous SEC Columns (7.8 mm × 300 mm), and a differential refractometric detector (Hitachi, Ltd., Japan). The eluent used was 0.1 M NaAc in HPLC-grade water, with a flow rate of 1 mL/min. The samples were dissolved in 0.1 M NaAc and filtered through a 0.45 μm filter before injection. The column temperature was 25 °C. The system was calibrated using linear dextran with Carbonic Anhydrase (MV 29000), Cytochrome c (MV 12327), T 10 (MV 10000), Subtilisin A (MV 7600), T 5 (MV 4600).

2.4. Monosaccharide identification

Monosaccharide composition was measured according to the following procedure: the polysaccharide was firstly dissolved into 5 mL 2 M trifluoroacetic acid, respectively, then solution was heated at 120 °C for 1 h. The supernatant was concentrated by rotary evaporation and then dried. The dried sample was dissolve into water and subjected to HPLC analysis. The following system was used: Rezex RPM-Monosaccharide Columns (7.8 mm × 300 mm), and a differential refractometric detector (Hitachi, Ltd., Japan). The eluent used was HPLC-grade water, with a flow rate of 0.4 mL/min. The column temperature was 80 °C. The following sugars were used as references: D-glucose, D-xylose, D-galactose, D-rhamnose, D-fructose (Fu, Tian, Cai, Liu, & Li, 2007).

2.5. Physical and chemical characters of purified polysaccharides

Examination of the characters of purified polysaccharides were determined as followings: the purified polysaccharides mixed with distilled water, ethnaol, methanol and acetone, respectively. Reducing sugar was determined by Fehling reagent (Schneider, 1979). Protein was measured by biuret reaction (Gornall, Bardawill, & David, 1949). Phenolic hydroxyls substance was assayed by the ferric chloride method (Zhou, 1978). Starchy polysaccharide was carried out by iodine reaction (Lee, Yu, & Chen, 1994). Uronic acids were assayed colorimetrically by the uronic acid–carbazole reaction (Bitter & Muir, 1962).

2.6. IR spectroscopy

The purified polysaccharides were ground with dry KBr powder (spectroscopic grade) and then pressed into a 1 mm pellet for FTIR measurement on an Nicolet Nexus 5DXC FT-IR infrared spectrometer (Nicolet Co. Ltd., USA) in the frequency range 4000–500 cm^{-1} (Kumar et al., 2004).

2.7. Antioxidant activity

2.7.1. Superoxide anion scavenging activity

The scavenging activities of purified polysaccharides for superoxide anion scavenging activity of various concentrations polysaccharide samples were determined based on the method of Marklund and Marklund (1974) with some modifications. 4.5 mL Tris–HCl buffer (0.05 M, pH 8.2) was kept in water bath at room temperature for 20 min. Then 1 mL of samples solution and 0.4 mL 1,2,3-phentriol (25 mM) were added and incubated at room temperature for 5 min. 1 mL HCl (8 mM) was added quickly to terminate reaction. The absorbance was read at 299 nm and the capability of scavenging the superoxide anion radicals were calculated according to the equation: Scavenging effect (%) = [1 − (Abs. of sample − Abs. of blank)/Abs. of control] × 100%.

2.7.2. Hydroxyl radical scavenging activity

Hydroxyl radical (radical HO•) can be generated via well-known Fenton-type reaction (Sminoff & Cumbes, 1989) with slight modification. Salicylic acid captures radical HO• to form 2,3-dihydroxyl benzoic acid, which had maximum absorbance at 510 nm (Xiong et al., 2011). The sample solution (0.1 mL) was mixed with 1.0 mL $FeSO_4$ (9 mM), 1.0 mL H_2O_2 (8.8 mM) and 1.0 mL salicylic acid (9 mM, dissolved by alcohol). 1.0 mL H_2O_2 (8.8 mM) was added to the reaction solution finally, and then the reaction mixture was incubated for 30 min at 25 °C. The absorbance of the mixture was measured at 510 nm. The scavenging activities of purified polysaccharides were calculated according to the equation: Scavenging effect (%) = [1 − (Abs. of sample − Abs. of blank)/Abs. of control] × 100%.

2.7.3. DPPH radicals scavenging activity

The DPPH radical scavenging activity was carried out using with the method described by Li, Zhou, and Han (2006) with minor modification. Briefly, 2 mL DPPH solution (0.2 mM in dehydrated alcohol) was added to 2.0 mL of related solution. After shaking vigorously, the mixture was left to stand for 30 min at 25 °C in the dark, and then the absorbance was measured at 517 nm. The inhibition of DPPH radicals by the samples was calculated as follows: Scavenging percentage activity on DPPH(%) = [1 − (Abs. of sample-Abs. of blank)/Abs. of control] × 100%.

3. Results and discussion

3.1. Extraction, separation and purification of the polysaccharides

The water-soluble crude polysaccharide (CP) was obtained as a light-yellow powder from the roots of *P. japonicus* rhizomes by enzyme method, ethanol precipitation, deproteinization, depigment and lyophilization.

Column chromatography results of CP are shown in Fig. 1. After fractionation on DEAE-Cellulose Cl^- column, CP-1, CP-2 and CP-3 (5.33:2.38:0.83) were obtained from an aqueous NaCl gradient (0,

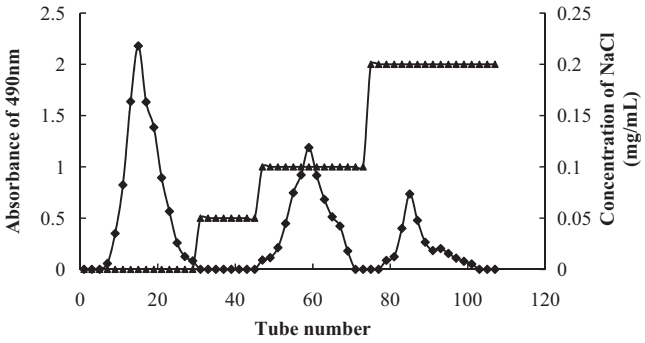

Fig. 1. DEAE-cellulose column elution profile of crude CP.

0.1,0.2 M). In view of the yield of CP-3 is least, it was not studied further. The two fractions of CP-1 and CP-2 were purified by a Sephadex G-75 column, respectively. Both CP-1 and CP-2 showed only one symmetrical peak in Fig. 2a and b. The main fraction was collected, cut-off by 3 kDa membrane, lyophilized and named as CP-1a and CP-2a for further antioxidant activity. Both of them appeared as a white powder.

3.2. Homogeneity, molecular weight determination and monosaccharide composition

The size-exclusion HPLC chromatography system was used to determine homogeneity and molecular weight of CP-1a and CP-2a. The homogeneity results are shown in Fig. 3a and b. Fig. 3a shows a single and symmetrical sharp peak, which indicates that CP-1a is a homogeneous polysaccharide. Fig. 3b shows two-peaks which implies CP-2a is not a homogeneous polysaccharide. The equation of the standard curve was: $\mathrm{Log}\, M_w = 6.1837 - 0.2274t$ (where M_w represents the molecular weight, while t represents retention time) with a correlation coefficient of 0.9996. The average molecular weight value of the purified polysaccharide CP-1a was estimated to be 6383 Da. CP-2a includes two different molecular weight polysaccharides which values were 6081 Da and 19920 Da. The monosaccharide composition of CP-1a and CP-2a was obtained after ion-exchange and gel permeation chromatography and analyzed qualitatively by HPLC. CP-1a and CP-2a were composed of glucose and galactose with a molar ratio of 15.02:1 and 14.38:1, respectively.

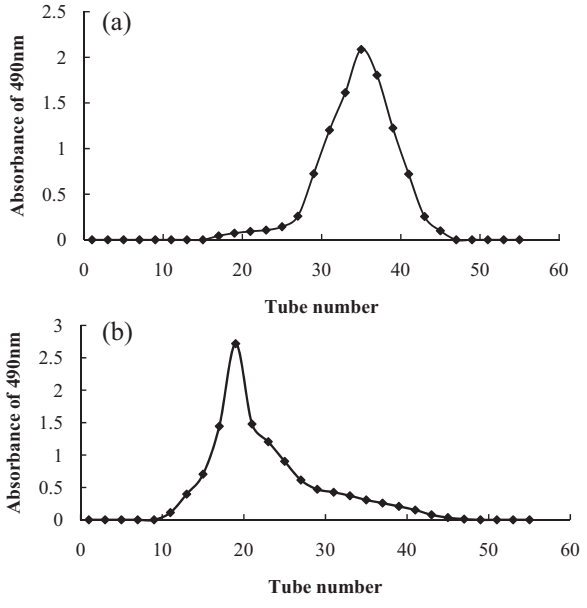

Fig. 2. (a) Sephadex G-75 column elution profile of CP-1 and (b) Sephadex G-75 column elution profile of CP-2.

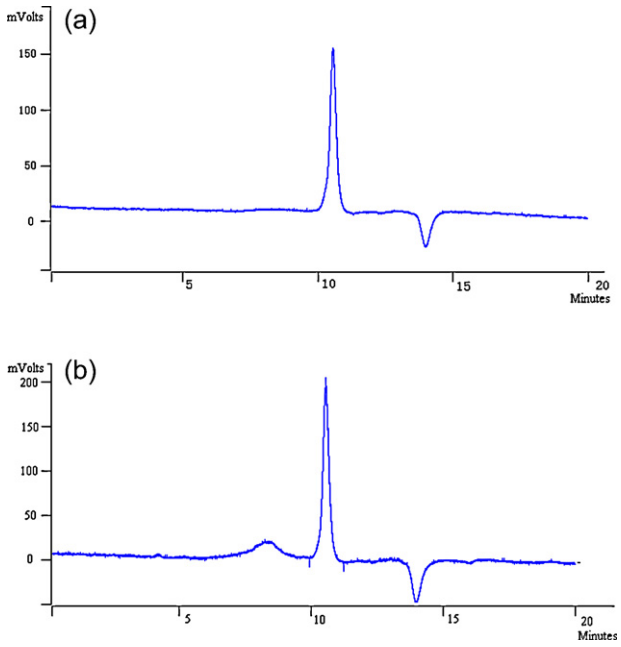

Fig. 3. (a) The profile of the CP-1a fraction in HPGPC and (b) the profile of the CP-2a fraction in HPGPC.

3.3. Physical and chemical characters of purified polysaccharides

Both CP-1a and CP-2a were dissolved in water, especially in hot water, which not dissolved in organic agents, such as ethanol, methanol and acetone. Fehling reagent methods indicate that no monosaccharides were present. Biuret reaction was minus reaction, which showed that protein was not present. The ferric chloride method showed that they did not contain phenolic hydroxyls substance. Iodine reaction proved that starchy polysaccharides were not present. The uronic acid–carbazole reaction were minus reaction, which indicated that both CP-1a and CP-2a did not contain uronic acids. The total sugar content of CP-1a and CP-2a was respectively determined to be 98.2% and 97.8% by the phenol-sulfuric method.

3.4. IR spectroscopy

In order to investigate the functional groups of the purified polysaccharides, the spectra were recorded at the absorbance mode from 4000 to 500 cm^{-1}. As shown in Fig. 4, the IR spectra of CP-1a and CP-2a was displayed a intensity and broad band at around 3430 cm^{-1}, which assigned to the hydroxyl groups stretching vibration. The weak intensity of band attributed to C–H group antisymmetrical stretching vibration (2930 cm^{-1}) could also be observed. The bands around 1570 cm^{-1} suggested the presence of the ester carbonyl groups (C=O). The absorptions at 1150 cm^{-1} and 926 cm^{-1} attributed to the stretching vibrations of pyranose ring. Both CP-1a and CP-2a were the polysaccharide which contains pyranose ring.

3.5. Antioxidant activity

3.5.1. Hydroxyl radical scavenging activities of CP-1a and CP-2a

Among the reactive oxygen species, hydroxyl radicals were the most harmful free radical and could induce severe damage to

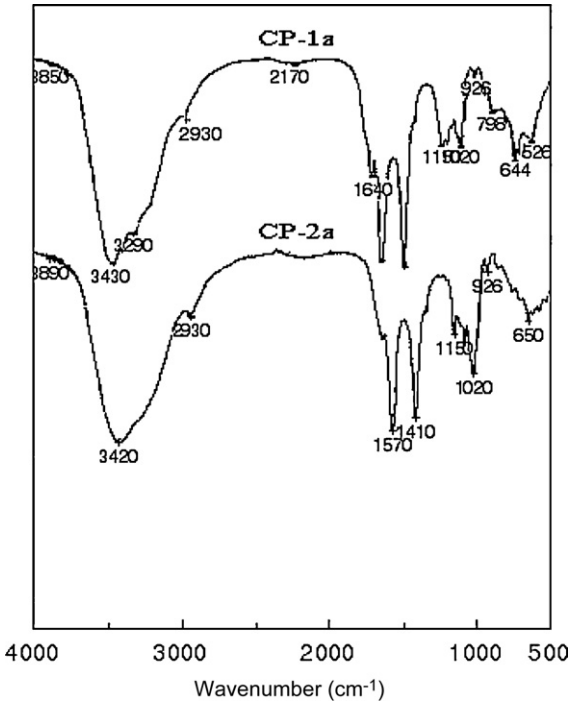

Fig. 4. IR spectroscopy of CP-1a and CP-2a.

adjacent biomolecules (Li et al., 2011). They were generated by reaction of Fe (II) complex with H_2O_2 in the presence of salicylic acid. Salicylic acid has the ability to absorb •OH to bring coloring material. Added hydroxyl radical scavengers compete with salicylic acid, which can pare coloring material down. The method was used to evaluate the hydroxyl radicals scavenging ability of natural compounds (Boligon, Pereira, Feltrin, & Machado, 2009). The effects of hydroxyl radical scavenging activities of CP-1a and CP-2a were given in Fig. 5. They showed have the activity of scavenging hydroxyl radicals. The scavenging activities of CP-1a and CP-2a increased with the increase of polysaccharide concentration ranging from 0.25 to 2 mg/mL, while it increased slowly at the concentration above 2 mg/mL. Compared CP-1a and CP-2a, CP-1a had a higher the scavenging ability, which is related to the different molecular weight between them (Zhao et al., 2006).

3.5.2. Superoxide anion scavenging activities of CP-1a and CP-2a

The superoxide radical, a relatively weak oxidant, is a highly toxic species which is generated by large numbers of biological and photochemical reactions (Banerjee, Dasgupta, & De, 2005). It decomposed to form stronger, reactive oxidative species and indirectly initiate lipid peroxidation as a result of H_2O_2 formation,

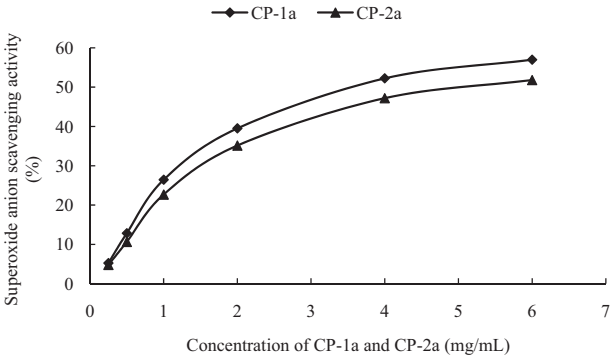

Fig. 6. Scavenging effects of CP-1a and CP-2a on superoxide anion.

creating precursors of hydroxyl radicals (Duh, Du, & Yen, 1999; Qi et al., 2006). Fig. 6 shows the scavenging activities of CP-1a and CP-2a against the superoxide radical. The scavenging effects of the two polysaccharides were well correlated with increased concentration up to 2.0 mg/mL. CP-2a had the weaker activity than CP-1a. At 4.0 mg/mL, the scavenging activities of CP-1a and CP-2a were 52.2% and 47.2%, respectively.

3.5.3. DPPH scavenging activities of CP-1a and CP-2a

The DPPH free radical has been widely used for evaluating the free radical scavenging activities of natural compounds (Amarowicz et al., 2004; Hu, Lu, Huang, & Ming, 2004). It shows a stable radical with a strong absorption band at 517 nm because of the odd electron. When the electron becomes paired off with electron of hydrogen atom donating previous antioxidant, the absorbance at 517 nm disappears (Annie, Arun, & Kuppusamy, 2006; Zhang et al., 2011). Fig. 7 describes the scavenging abilities of CP-1a and CP-2a from *Panax japonicas* on DPPH radical. It showed that the scavenging abilities of polysaccharides on inhibition of the DPPH radical were related to the concentration of the samples. The higher concentration the higher level of scavenging ability was found for all samples used in the test. At 0.5 mg/mL, CP-1a showed scavenging abilities of 48.9% on DPPH radicals. At 4 mg/mL, the scavenging abilities increased to 93.2%. CP-2a exhibited a little lower radical scavenging activity than CP-2a at the same concentration point. The results mentioned above suggested that CP-1a and CP-2a might act as electron or hydrogen donator to scavenge DPPH free radical. Comparison of response to the previous antioxidant capacity value among three assays revealed that DPPH assay exhibited the highest value (compared to superoxide anion and hydroxyl radicals scavenging activities assays).

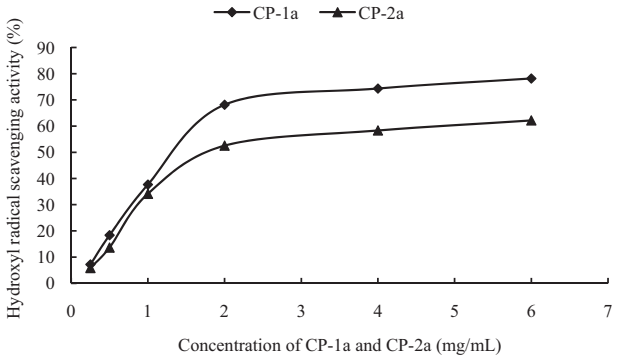

Fig. 5. Scavenging effects of CP-1a and CP-2a on hydroxyl radical.

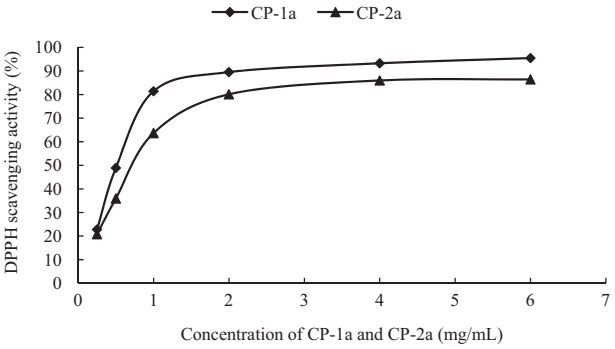

Fig. 7. Scavenging effects of CP-1a and CP-2a on DPPH.

4. Conclusions

This article mainly related to the isolation of the antioxidant activities polysaccharides. According to the results stated above, it could be concluded that we obtained two polysaccharide fractions purified by DEAE Cellulose and Sephadex G-75 column chromatography. In vitro antioxidant activities studies indicated both CP-1a and CP-2a exhibit strong activity in scavenging free radicals. Various factors influenced the antioxidant activity of *P. japonicus* polysaccharides. Thus, further research is needed to explore the complete structure, conformation and mechanism of antioxidant activity.

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