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Full Length Research Paper

Isolation, chemical composition and antioxidant activities of a water-soluble polysaccharide from rhizoma atractylodis macrocephalae

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A water-soluble polysaccharide extracted from Rhizoma Atractylodis Macrocephalae was fractioned into two fractions coded as F1 and F2. Structure features of the purified polysaccharides were investigated by a combination of chemical and instrumental analysis, such as gas chromatography-mass spectrum (GC-MS) and fourier transform-infrared spectroscopy (FTIR). The results indicated that the F1 was composed of galactose, rhamnose, arabinose and mannose. F2 contained xylose, galactose and arabinose. Two fractions showed high antioxidant activities. Moreover, antioxidant activities of F2 is stronger.

Key words: Rhizoma atractylodis macrocephalae, polysaccharides, antioxidant.

INTRODUCTION

The presence of free radicals in biological materials was discovered less than 50 years ago (Droge, 2002; Vasu et al., 2009). A free radical can be defined as an atom or a molecule with an unpaired electron. The presence of the unpaired electron makes certain free radicals highly reactive with nearby chemical species and potentially dangerous to living tissue. Excess free radicals can result from tissue damage and hypoxia, overexposure to environmental factors (smoking, ultraviolet radiation, and pollutants), a lack of antioxidants, or destruction of free radical scavengers. It is now accepted that free radical-mediated oxidation of biological molecules such as lipids, proteins, and DNA is involved in a variety of disorders and diseases (Halliwell and Gutteridge, 2007; Trombino et al., 2009). Above all, lipids are very susceptible to free radical attack and lipid peroxidation induces alterations in integrity, disturbances in fine structure, and functional loss of biomembranes (Niki, 2009; Alim et al., 2009). Furthermore, lipid peroxidation mediated by free radicals proceeds by a chain mechanism, amplifying the damaging effect of free radicals (Niki, 2009; Yin and Porter, 2005; Yan et al., 2011). Lipid peroxidation products are potentially cytotoxic and modify proteins and DNA (Marnett et al., 2003; Liu et al., 2010). In addition to natural antioxidants such as vitamin C, vitamin E, carotenoids, and flavonoids (Ares et al., 2009), a number of natural antioxidants have been assessed for prevention of lipid peroxidation in various systems (Arabshahi-D et al., 2007; Wang et al., 2009).

Rhizoma Atractylodis Macrocephalae is a plant in family of compositae with a plenty of natural resource in Zhejiang, Jiangsu and Anhui Provinces in China. The rhizoma atractylodis macrocephalae Koidz. (RAM) as a traditional Chinese medicine has been utilized for at least 2000 years. The annually consumed RAM for medical purpose is estimated at 7000 tons in this country (Chen et al., 2007).

The RAM is often cooked together with other herbs and the soup is used for oral administration in the treatment of diseases such as diarrhea and infections in humans and animals. For examples, Zhou (2001) has reported effective treatment of infections in the upper respiratory tract in 125 cases with Shen Ling Bai Zhu San, which contains rhizoma atractylodis macrocephalae, Radix Codonopsis Pilosulae, Poria, Radix Glycyrrhizae, Rhizoma Dioscorea, Semen Dolichoris Album, Semen Nelumbinis, Semen Coicis, Fructus Amomi and Radix

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Platycodi. In this study, polysaccharides isolated from Rhizoma Atractylodis Macrocephalae were divided into two fractions (F1 and F2). Their chemical structure and antioxidant activities were evaluated.

MATERIALS AND METHODS

Extraction of polysaccharides

Rhizoma Atractylodis Macrocephalae was purchased from a local herb shop in Taizhou city, China. Rhizoma Atractylodis Macrocephalae samples (20 g) were freeze-thawed and washed in pure water, followed by lyophilization, and were milled into small pieces. The milled samples were washed three times using a large amount of ethanol with shaking (120 rpm) overnight, and then collected by filtration using gauze. The ethanol-washed samples were put into distilled water (300 ml) at 100°C, and agitated at constant temperature for 4 h to yield the transparent solution. The solution was filtrated. Then the filtrate was concentrated by rotary evaporator to create a highly viscous solution. The viscous solution (60 ml) was slowly poured into 100% ethanol (1000 ml) to precipitate polysaccharides.

Separation and purification of rhizoma atractylodis macrocephalae polysaccharides

One milliliter of 1 mg/ml RAMP was loaded onto a Sephadex G-100 gel column (10 x 500 mm), eluted with 25 ml of distilled water at a flow rate of 0.2 ml/min. Each fraction with 1 ml of eluate was collected. All these fractions were determined by the phenol-sulphuric acid method. The chromatography profile was drawn by Microsoft Excel 2000 (Microsoft, Seattle, WA). The peak with the highest polysaccharide content was collected and then freeze-dried.

Monosaccharide/alditol composition analysis

Samples were prepared as described previously (Kim et al., 2005). Briefly, dried samples were methanolyzed with 3 N HCl in MeOH for 2 h at 121°C, followed by re-N-acetylation and trimethylsilylation.

The gas chromatography (GC) apparatus used was a Hewlett-Packard HP5890 with a fused silica capillary column (30 m x 0.25 mm I.D, 0.25 µm film thickness) and SPB-5 (SUPELCO). Helium gas was used as a carrier gas, and the column was heated at 60°C for 1 min and then at 8°C/min to 280°C.

Inlet and detector (FID) temperatures were 280°C, injection mode was splitless, and injection volume was 1 µl. The gas chromatography – mass spectrum (GC-MS) apparatus used was a JEOL Ltd JMS-700 V and Agilent 6890 with a fused silica capillary column (30 m x 0.25 mm I.D) and SPB-5(SUPELCO). Helium gas was used as a carrier gas, the column was heated at 60°C for 1 min and then at 8°C/min to 280°C. Inlet and detector (FID) temperatures were 280°C.

Injection mode was splitless, and injection volume was 1 µl. The ionizing mode was EI, ionizing energy was 70 eV, ionizing current was 100 µA, temperature was 280°C, accelerating voltage was 10 kV, and scan rate was 1 s/scan (m/z 35–500).

FT-IR analysis

The lyophilized samples were measured by fourier transform-infrared spectroscopy (FT-IR) as a film between two KBr plates on a Shimadzu IR-8000 spectrophotometer.

DPPH assay

The method used by Hatano et al. (1989) for the measurement of the antioxidant activity of polysaccharides was followed in this study. In the presence of an antioxidant, the purple color of DPPH decays and the change of absorbance can be followed spectrophotometrically at 517 nm. In addition, 1.5 ml of a 0.1 mM of DPPH in methanol was mixed with equal volume of polysaccharides extract in methanol, mixed well and kept in dark for 50 min, and the activity was measured spectrophotometrically at 517 nm in the presence of different concentration of samples (0.5 to 1.2 mg ml^{-1}). Blank experiment was also carried out to determine the absorbance of DPPH without any sample.

Scavenging activity in this assay was expressed as IC50, which represents the concentration of the extract (mg/ml) required to inhibit 50% of the free radical-scavenging activity. Each value in the table was obtained by calculating the average of three determinations ± standard deviation.

Animals, diet manipulation and RAMP treatment

Male Wistar rats, at 7 weeks of age, were used in this experiment and maintained under conventional conditions. Thirty-two rats were divided into four groups: normal group (low fat diet), control group (high fat diet) and two polysaccharides-treated groups consuming high fat diets, n = 8 per group. Rats were fed the normal (low fat) diet (group 1) or the high fat diet (groups 2 to 4) for 6 weeks. High fat diet composition was prepared according to method (Kim et al., 2004) (Table 1).

Rats were administered orally once daily with F1 (group 3) and F2 (group 4) at the dose of 200 mg/kg body weight for 5 weeks from the 1st day of the high fat diet feeding, PBS was orally administered to the rats in the model control group (group 2).

Biochemical analysis

The plasma thus obtained was used for malondialdehyde (MDA) (product of lipid peroxidation) estimation. Plasma MDA was measured by a thiobarbituric acid assay procedure (McCord and Fridovich, 1969), which was calibrated using 1, 1, 3, 3 tetraethoxypropane (Sigma Chemicals, St. Louis, MO, USA) as a standard. Results were expressed as nanomoles of MDA per millimeter of serum. Superoxide dismutase activity was measured by using commercial kits (Biovision, K335-100).

The activity was expressed as U/ml. Catalase (CAT) was measured by the method of Sinha (1972) and glutathione peroxidase (GPx) was by the method of Rotruck et al. (1973). GSH was estimated by the method of Moron et al. (1979).

Statistical analyses

Data were analyzed using Student's t-test and $p < 0.05$ was considered significant. Values are expressed as means ± standard deviation (S.D.) for eight rats per group.

RESULTS AND DISCUSSION

Purification of the polysaccharides

A dialysed fraction of crude polysaccharides from Rhizoma Atractylodis Macrocephalae polysaccharides, cut-off by 14 kDa membrane, was chromatographed on a

Table 1. Compositions of the experimental diet (g/kg diet).

Ingredients	Groups	
	Normal	High fat diet
Casein	200.0	200.0
DL-Methionine	3.0	3.0
Cornstarch	650.0	150.0
Sucrose	–	150.0
Cellulose	50.0	50.0
Corn oil	50.0	–
Beef tallow	–	400.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Choline bitarate	2.0	2.0
Fat energy (%)	5.0	40.0
Total (g)	1000.0	1000.0

Normal diet: AIN-76A purified rodent diet with 65% cornstarch #111753CLL (Dyets Inc., Bethlehem, PA, USA); High fat diet: 40% beef tallow modified AIN-76A purified rodent diet #101556 (Dyets Inc., Bethlehem, PA, USA).

Sephadex G-100 gel column (10 x 500 mm). After Sephadex G-100 gel column (10 x 500 mm) separation, the two separated fractions (F1 and F2) of RAMP were collected as 5 ml fractions by the automated fraction collector, then, concentrated, dialysed and lyophilized (Figure 1).

Chemical composition of the polysaccharides

The purified F1 and F2 were hydrolyzed by TFA into individual monosaccharides that were further trimethylsilylated for gas chromatography analysis. The results were summarized. Four monosaccharides, including galactose, rhamnose, arabinose and mannose, were identified for F1 after comparison with the monosaccharide standards. Their molar percentages were 3.9, 14.3, 42.7 and 26.6%, respectively. Three monosaccharides, including xylose, galactose and arabinose, were identified for F2 after comparison with the monosaccharide standards. Their molar percentages were 11.5, 34.1 and 44.2%, respectively.

FT-IR analysis of the polysaccharides

The bands in the region of 3398.32 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2972.73 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1703.15 cm^{-1} were due to associated water. Moreover, the characteristic absorptions at 834.24 and 895.51 cm^{-1} in the IR spectra indicated α- and β-configurations simultaneously existing in Rhizoma Atractylodis Macrocephalae polysaccharides.

DPPH radical scavenging activity of F1 and F2

Antioxidants are considered important nutraceuticals on account of many health benefits (Droge, 2002; Lee et al., 2004; Valko et al., 2007). The requirement of a standard assay is very important in order to compare the results of different laboratories and validation of the conclusions. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund et al., 2005). Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay (Alma et al., 2003; Karioti et al., 2004; Kordali et al., 2005). A perusal of the publications in the recent past (Table 1) shows that various research groups have used widely different protocols which differed in the concentration of DPPH (22.5 to 250 µM), incubation time (5 min^{-1} h), reaction solvent and pH of the reaction mixture. High concentrations of DPPH in the reaction mixture give absorbance beyond the accuracy of spectrophotometric measurements (Ayres, 1949; Sloane and William, 1977) Figure 2 showed the percentage inhibition of DPPH radical generation of F1 and F2. The extract (F1 and F2) exhibited effective antioxidant activity at all concentrations. The antioxidant activity of F1 and F2 increased with increasing concentration. The all of doses of F2 showed higher antioxidant activities than F1.

In vivo antioxidant activity

Lipid peroxidation in lungs is measured by the commonly used method called the thiobarbituric acid test (TBARS) (e.g., Oakes and Van Der Kraak, 2003; Almroth et al.,

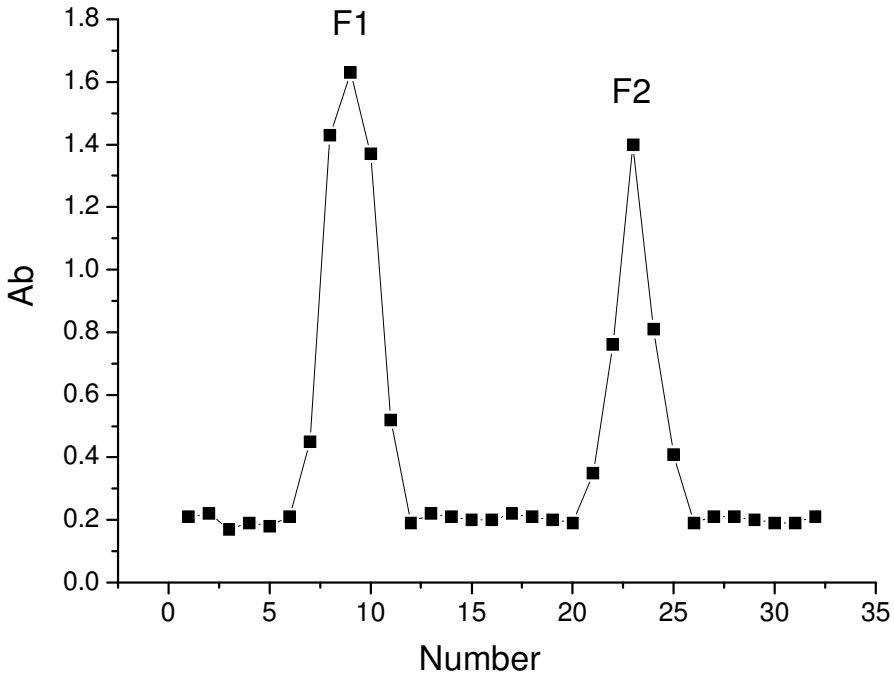

Figure 1. Elution curve of polysaccharides (F1 and F2)from membrane separation by Sephadex G-100 gel column (10x 500 mm).

2005), which relies on the ability of polyunsaturated fatty acids (PUFAs) of cell membranes to readily react with oxygen radicals by donating a hydrogen atom. The fatty acid radical is unstable and a chain of reactions occur, of which MDA is one of the end products. Peroxidation of membrane lipids affects fluidity between intra- and extra-cellular environments, integrity of other molecules in the cell membrane (proteins and cholesterols) and, if the perturbation is severe, the cell wall collapses causing apoptosis.

Reduced glutathione (GSH) is a ubiquitous tripeptide produced by plants and animals alike from the amino acids glutamine, glycine, and cysteine (with cysteine being the rate-limiting constituent). Its sulfur-hydrogen, or thiol, group is a potent reducing agent, and GSH can be considered the one of the body's most important water-soluble antioxidants.

When blood TBARS and GSH concentrations in rats treated with and without F1 and F2 were determined, the results shown in Table 2 were obtained. There were significantly changes in blood TBARS and GSH concentrations between group 1 and group 2. However, the medicine-treated groups had significantly lower blood TBARS concentration and significantly higher blood GSH concentration than the untreated control group. In the present study, neither plasma TBARS nor blood GSH concentrations were affected by fluctuation in ovarian hormone plasma concentrations, and these results are in agreement with previous data (Chung et al., 1999).

A number of methods have been established as markers of oxidative stress in animal cells, caused either by excessive production of ROS or reduced antioxidant defense (Armstrong, 2002). Superoxide dismutases (SOD) are metalloenzymes that catalyze the dismutation of superoxide anion ($\bullet O_2^-$) into O_2 and hydrogen peroxide (H_2O_2) (Fridovich, 1986). The Cu/Zn-SOD is localized in the cytosol and nucleus, while Mn-SOD is located within the mitochondrial matrix. Subsequently, H_2O_2 is reduced to H_2O by glutathione peroxidase (GSH-Px) in the cytosol, or by catalase (CAT) in the peroxisomes. SOD, CAT and GSH-Px, together with GSH-S-transferase and GSH reductase, are easily induced by oxidative stress, and the activity levels of these enzymes have been used to quantify oxidative stress in cells (Van der Oost et al., 2003). Glutathione and GSH disulfide (GSSG) are biologically important intracellular thiols, and alterations in the ratio between total GSH (tGSH) and GSSG (oxidative stress index) are also often used to assess exposure of cells to oxidative stress.

The SOD, CAT and GSH-Px activities in tissue homogenates were significantly decreased in high-fat rats comparing with controls. The administration of F1 and F2 to high fat rats increased significantly ($p < 0.01$) serum SOD, CAT and GSH-Px activities (Table 3).

Conclusion

Rhizoma Atractylodis Macrocephalae polysaccharides

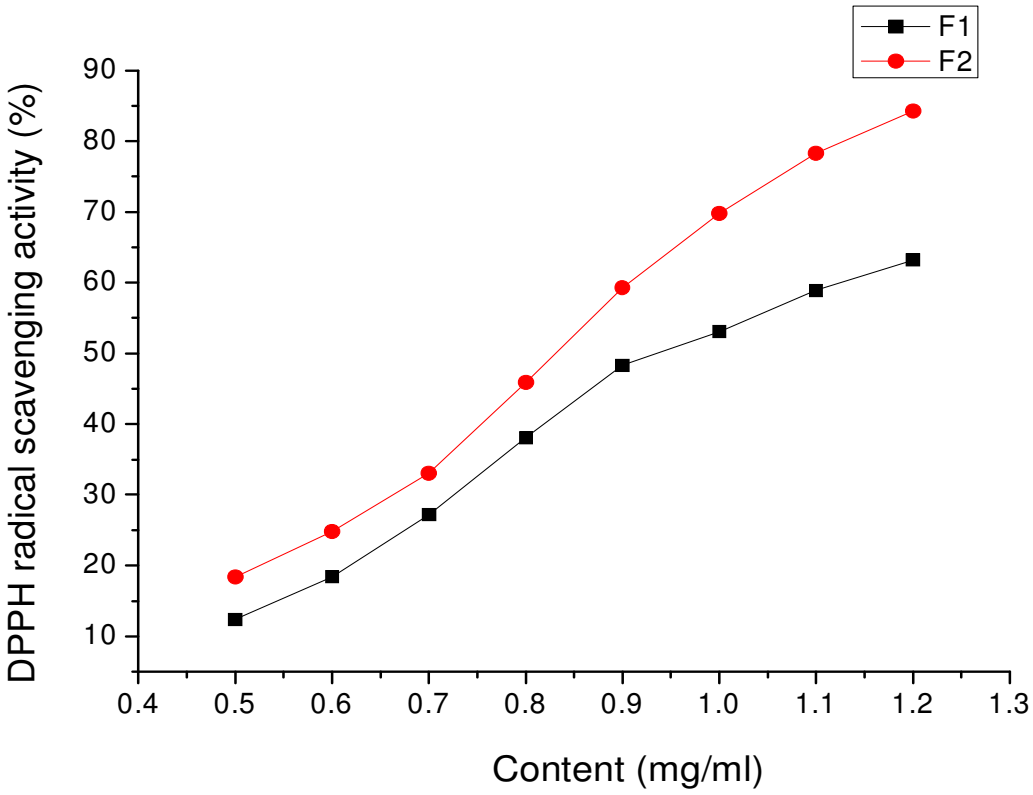

Figure 2. DPPH radical scavenging activity of different contents of F1 and F2.

Table 2. Effect of F1 and F2 on blood TBARS and GSH levels in rats.

Group	TBARS (μmol/ml)	GSH (μmol/ml)
1	0.14 ± 0.02	40.83 ± 2.94
2	0.31 ± 0.04 [b]	21.53 ± 1.47 [b]
3	0.21 ± 0.03 [d]	31.54 ± 2.41 [d]
4	0.18 ± 0.02 [d]	38.41 ± 1.72 [d]

[b]$p < 0.01$, compared with group 1, [d]$p < 0.01$, compared with group 2.

Table 3. Effect of F1 and F2 on blood SOD, CAT and GSH-Px levels in rats.

Group	SOD	CAT	GSH-Px
1	194.3 ± 10.2	23.12 ± 1.01	17.43 ± 0.98
2	89.3 ± 4.2 [b]	13.11 ± 1.23 [b]	10.32 ± 0.56 [b]
3	132.1 ± 9.4 [d]	17.83 ± 1.06 [d]	14.82 ± 0.62 [d]
4	173.2 ± 11.3 [d]	21.94 ± 1.85 [d]	15.81 ± 0.94 [d]

[b]$p < 0.01$, compared with group 1, [d]$p < 0.01$, compared with group 2.

were divided into two fractions (F1 and F2). Chemical composition and structure of F1 and F2 were analyzed and some characteristic structure of polysaccharides were shown. Pharmacological analysis showed that F1 and F2 possessed strong antioxidant activities *in vitro* and *in vivo*. Moreover, F2 displayed stronger antioxidant

activity than F1.

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